VOLUME 38 AUGUST 1960 NUMBER 8

Canadian Journal of Biochemistry and Physiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Biochemistry and Physiology

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COMPOSITION OF EPIPHYSEAL CARTILAGE

I. CHANGES IN HEXOSAMINE AND ACETONE EXTRACTABLE CONTENTS OF EPIPHYSEAL CARTILAGE OF RACHITIC CHICKS FOLLOWING ADMINISTRATION OF VITAMIN D_3[1]

J. D. CIPERA, B. B. MIGICOVSKY, AND L. F. BÉLANGER[2]

Abstract

The effect of vitamin D on the components of epiphyseal cartilage was investigated. Three-week-old rachitic chicks were pooled into two groups, one of which received vitamin D_3, while the other served as a control. The epiphyseal cartilage from these two groups was subjected to comparative study which is described in detail. Two distinct effects that resulted from the vitamin D_3 administration were the significant increases in the hexosamine content and in the amount of acetone-extractable solids.

Histological studies of the epiphysis of rachitic chicks (1) showed extensive alterations in the morphology of the epiphyseal cartilage 24 and 48 hours after vitamin D_3 administration. In order to ascertain the nature of chemical processes which accompanied these alterations we investigated several components of epiphyseal cartilage. Our interest centered on chemical changes in the cartilage occurring at the stage when vitamin D_3 was beginning to take effect, but the calcification process was not yet noticeable. Such changes might conceivably take place in the organic portion of the epiphyseal cartilage.

The predominant organic components of cartilage are collagen and chondroitin sulphate (2). Chondroitin sulphate has long been suspected to be a prime factor in the calcification process, due to the presence of regularly spaced strongly anionic groups (3, 4, 5). A distinguishing characteristic is its high content of N-acetyl-2-amino-2-deoxy-D-galactose (6, 7). Therefore we started our studies with investigation of relative hexosamine contents of cartilage.

Because the epiphyseal cartilage is a dynamically changing tissue in which the various stages of development are often interdispersed and not clearly differentiated, the isolation of reproducible aliquots from tibiae requires special attention. The literature offers scant information on this problem. Most workers used other types of cartilage, e.g. nasal septa (8, 9, 10, 11), which do not undergo the changes typical of epiphyseal cartilage, are consequently quite homogeneous, and lend themselves to the preparation of reproducible aliquots.

[1]Manuscript received February 12, 1960.
Contribution No. 24, from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.
[2]Department of Histology and Embryology, School of Medicine, University of Ottawa, Ottawa, Ontario.

Attempts to include epiphyseal cartilage in these investigations have yielded inconclusive results because of variability of samples (12).

While investigating the means of dissecting the epiphyseal tissues, we noticed an interesting difference in the manner in which freezing affected the zones (1) in the epiphysis. The articular cartilage remained homogeneous in appearance at room temperature and in the frozen state. The epiphyseal cartilage, on the other hand, appeared homogeneous at room temperature only. In the frozen state it had the same gross appearance as the underlying tissue, i.e., the visual differentiation present at room temperature was obscured.

Our procedure for isolating the epiphyseal cartilage from the chicken tibiae, which is described in the experimental section, yielded fairly reproducible aliquots. Analytical investigations of these aliquots revealed a significant increase in the hexosamine content of the cartilage upon the administration of vitamin D_3. For example, analyses of hydrolyzates of epiphyseal cartilage from 3-week-old rachitic chicks indicated that the hexosamine content of dried cartilage was $5.98 \pm 0.196\%$[3] as compared with $7.24 \pm 0.185\%$[3] in cartilage from similar chicks that had received 5000 I.U. vitamin D_3 the previous day.

In order to prove the significance of these preliminary results we set up a carefully controlled experiment. Tibiae from vitamin D_3-treated and untreated chicks were divided into several groups and analyzed separately for their components.[4] We used this experiment also to investigate the interaction of sex and vitamin D_3 treatment on the composition of cartilage tissues.

Materials and Methods

Animals

One-day-old chicks were fed A.O.A.C. (13) low calcium rachitogenic diet for 3 weeks. They were then divided into two groups of 100 chicks each. For the next 2 days the chicks in the first group received a daily oral dose of 2000 units of vitamin D_3, while those in the second group were maintained on the rachitogenic diet without change. At the end of the 2-day treatment period all the chicks were killed and both tibiae were excised. The chicks were sexed and the tibiae from each group were separated into eight subgroups, four male and four female. Each group consisted of 20 to 30 tibiae.

Isolation of Epiphyseal Cartilage

Only epiphyseal cartilage from the proximal end of the tibiae was dissected because the cartilage from the distal end is difficult to cut out cleanly. The proximal ends were split sagittally, the articular cartilage was discarded, and, under low magnification, the remaining epiphyseal cartilage was carefully dissected.

Drying and Defatting the Cartilage

The raw epiphyseal cartilage was weighed immediately after dissection and placed under acetone in a cold-room (0–3° C), where it was homogenized in acetone at high speed, using the VirTis 45 homogenizer, for approximately

[3]Each mean ± S.E. represents analytical values of four aliquots.
[4]The vitamin-D_3-treated groups will be designated as "positive" and untreated as "negative".

15 minutes. The dispersed cartilage was transferred by decanting the supernate into 50-ml centrifuge tubes, fresh acetone was added to the cartilage remaining in the homogenizer, and the procedure was repeated until all cartilage was homogenized. The homogenized cartilage was then isolated by centrifugation, washed twice with acetone, air-dried, and finally placed *in vacuo* over phosphorus pentoxide. Hereafter the dried and defatted cartilage is referred to as "dried" cartilage.

The acetone phase and washings were collected and evaporated, and the weight of the residue recorded as acetone-extractable solids.

Ashing

"Dried" cartilage aliquots were ashed in platinum crucibles at 800° C to constant weight. Vycor crucibles were unsuitable because of corrosion, which produced an "ash" insoluble in acids.

Analysis of Ash

In order to bring the ashed aliquots into solution quantitatively they were treated twice with 0.5 ml of concentrated hydrochloric acid (removing the hydrochloric acid each time by carefully warming the crucibles on a hot plate until the aliquots were dry again), then dissolved in 0.1 *N* hydrochloric acid and transferred quantitatively into 1-ml volumetric flasks, using 0.1 *N* hydrochloric acid to wash the walls of the crucible and to make up to full volume.

Aliquots of this solution were used for the analysis of calcium by the classical oxalate–permanganate method (14).

Hydrolysis of Cartilage

Aliquots of "dried" cartilage were hydrolyzed by 4 *N* hydrochloric acid in sealed glass tubes at 100° C for 12 hours. The tubes were then quickly cooled, the minute amounts of undissolved residues were pushed to one side of the tubes by centrifugation, and the dark brown hydrolyzates pipetted out and immediately evaporated to dryness *in vacuo* (40° C bath temperature). Traces of hydrochloric acid were removed under high vacuum over potassium hydroxide.

Estimation of Hexosamines in the Hydrolyzates

The hydrolyzates were each dissolved in 5 ml distilled water, and 1-ml aliquots of these solutions were used for the estimation of hexosamine content. The aliquots were made up to 25 ml with water and decolorized with Norite, and the filtrates were further treated by the Schloss modification of the classical Elson-Morgan method (15).

Results and Discussion

Group averages of 120 to 150 mg of raw epiphyseal cartilage and 30 to 40 mg of "dried" cartilage per tibia were isolated.

Table I shows there was little difference in the yields of either raw or "dried" cartilage from positive and negative chicks. On the other hand, there was a considerable difference in the amount of acetone-extractable solids, the positive cartilage yielding almost twice as much of this material as the negative cartilage.

TABLE I

Cartilage fractions isolated from tibiae

Type of tibiae	No. groups*	Raw cartilage, mg/tibia ± S.E.	"Dried" cartilage, mg/tibia ± S.E.	Acetone extract	
				mg/tibia ±S.E.	% "dried" cartilage
Negative male	4	154± 7.6	37.8±3.58	1.5 ±0.04	3.97
Negative female	4	131± 6.45	34.6±1.98	1.45±0.26	4.37
Positive male	3	135± 4.7	33.6±0.72	2.5 ±0.21	7.55
Positive female	3	131±15.4	31.6±4.75	2.5 ±0.09	8.30

*Each group represents the pooled epiphyseal cartilage from approximately 25 tibiae.

"Dried" epiphyseal cartilage yielded 10–12% ash, the subgroups of positive cartilage having a slightly higher mean ash value (11.4%) than the negative (10.8%), and the male (11.4%) higher than female (10.7%). These differences do not appear to be significant.

Calcium contents of ash were quite uniform, no significant differences being apparent between the negative and positive, or between the male and female groups.

The degradation of organic polymers was accomplished by an acid hydrolysis. All the aliquots behaved in a similar manner on hydrolysis, producing a brown, water-soluble, sticky substance, with an aroma reminiscent of a meat extract, and a minute amount of a black, finely dispersed, insoluble residue. The weights of insoluble residues varied within the range of 5–8% (of the weight of "dried" cartilage), while the weights of soluble hydrolyzates were 115–125%.

The difference in weight between "dried" cartilage and soluble hydrolyzate could be indicative of the relative amount of water used up for the hydrolysis of the polymers, providing that reactions between hydrochloric acid and the mineral components of the cartilage did not cause significant weight variations. Since in this series of analyses the difference in the average weight increase of hydrolyzate from the positive groups (20.5%), as compared with negative (17%), was relatively only slightly higher than the difference in the yield of ash (11.4% positive; 10.8% negative), we have drawn no conclusions at this stage of our investigations.

The results of the analysis for hexosamines, which are shown in Table II, fully confirmed the results of our preliminary investigations. The increase in

TABLE II

Hexosamine content of epiphyseal cartilage

Treatment	Group No.	Male	Female	Hexosamines, % of "dried" cartilage, mean ± S.E.
No vitamin D_3	1	7.24	7.02	
	2	7.22	7.14	
	3	5.99	5.97	
	4	5.65	6.29	6.57±0.220
Two doses of 2000 I.U. of vitamin D_3 48 hr and 24 hr before killing	5	7.47	7.83	
	6	8.21	8.23	
	7	7.69	7.37	7.80±0.135

the hexosamine content of the epiphyseal cartilage of the chicks as a consequence of administration of vitamin D_3 may be indicative of the histological changes observed by Bélanger and Migicovsky (1).

References

1. L. F. BÉLANGER and B. B. MIGICOVSKY. J. Exptl. Med. **107**, 821 (1958).
2. J. E. EASTOE. *In* The biochemistry and physiology of bone. *Edited by* G. R. Bourne. Academic Press, Inc., New York. 1956.
3. E. E. BOYD and W. F. NEUMAN. J. Biol. Chem. **193**, 243 (1951).
4. S. J. FARBER and M. SCHUBERT. J. Clin. Invest. **36**, 1715 (1959).
5. J. R. DUNSTONE. Biochem. J. **72**, 465 (1959).
6. S. P. JAMES, F. SMITH, M. STACEY, and L. F. WIGGINS. J. Chem. Soc. 625 (1946).
7. K. MEYER, P. HOFFMAN, and A. LINKER. *In* Connective tissue symposium. *Edited by* R. E. Tunbridge. Charles C. Thomas, Publisher, Springfield, Ill. 1957. p. 86.
8. H. G. BRAY, J. E. GREGORY, and M. STACEY. Biochem. J. **38**, 142 (1944).
9. S. M. PARTRIDGE. Biochem. J. **43**, 387 (1948).
10. M. B. MATHEWS and A. DORFMAN. Arch. Biochem. Biophys. **42**, 41 (1953).
11. R. V. WEBBER and S. T. BAYLEY. Can. J. Biochem. and Physiol. **34**, 993 (1956).
12. G. SMITS. Biochim et Biophys. Acta, **25**, 542 (1957).
13. THE ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS, Washington, D. C. Methods of analysis. 5th ed. 1940. p. 371.
14. E. P. CLARK and J. B. COLLIP. J. Biol. Chem. **63**, 461 (1925)
15. B. SCHLOSS. Anal. Chem. **23**, 1321 (1951).

THE EFFECT OF THE PROLONGED INTAKE OF ETHYLENEDIAMINETETRAACETATE ON THE UTILIZATION OF CALCIUM AND IRON BY THE RAT[1]

B. A. LARSEN,[2] W. W. HAWKINS, VERNA G. LEONARD, AND JOAN E. ARMSTRONG

Abstract

The dihydrate of disodium ethylenediaminetetraacetate (EDTA) was added at levels of 0.3 and 1.5% to the diet of young rats to produce molar ratios EDTA:Ca:Fe of 1:49:0.8 and 1:9.8:0.16. These diets did not affect the excretion of calcium or its concentration in blood serum or femur. In both cases the concentration of iron in serum was increased, and in spleen decreased; and with the higher dietary level of EDTA the concentration of iron in the liver was decreased. Other animals were given 0.5 or 3.0 mg of the EDTA compound per day intraperitoneally. The only difference in any of the same measurements noted above was a decrease in the concentration of iron in the spleen in the animals which received the larger dose. There was no effect in any case on the size of the liver, spleen, or femur, or upon growth. All the measurements were made after 110 – 112 days. The larger amounts of EDTA which were used were near the maxima which the animals would tolerate for a long period.

Introduction

An introduction to the subject of this paper may be found in a previous one from this laboratory (1).

Ethylenediaminetetraacetic acid (EDTA) and some of its salts are sequestering agents, and can be used in food processing when the presence of certain metals is responsible for an undesirable color. In the animal body EDTA can modify the absorption and metabolism of calcium and iron, so that deficiencies could possibly occur (see references in (1)).

We previously demonstrated (1) in the rat that when Fe^{59} was fed with EDTA there was a greater excretion of the metal in both faeces and urine, and less in representative body organs, including the spleen and liver. Apparently when EDTA is ingested, less iron is absorbed, and more of what is absorbed is excreted. When the EDTA was fed after the Fe^{59} had been absorbed, the Fe^{59} content of the liver decreased, and that of the blood and spleen increased as its urinary excretion increased, so that less was available for metabolic purposes.

Those experiments were short-term. The present one was designed as an extension of them, and to include observations on calcium. It sought the demonstration of effects on the utilization of calcium and iron after the prolonged intake of EDTA.

Procedure

Young albino rats were separated into five groups (A–E) of 20 animals each, with the sexes equally divided, and with an average individual body

[1]Manuscript received March 9, 1960.

Contribution from the Atlantic Regional Laboratory, National Research Council, Halifax, N. S. This paper was presented at the third annual meeting of the Nutrition Society of Canada in Guelph, Ontario, on June 24, 1960.

Issued as N. R. C. No. 5747.

[2]Postdoctorate Fellow, National Research Council of Canada, 1958–59. Present address: Norwegian Institute of Seaweed Research, NHT, Trondheim, Norway.

weight of 85 g. They were kept individually in screen-bottom cages. Water was allowed ad libitum. The basal diet was powdered fox chow. All were fed the same amount each day, 10–15 g per rat, which was sufficient to support a good rate of growth (see Fig. 1).

The animals in group A were not given EDTA. Those in groups B and C were given respectively 0.5 and 3.0 mg of the disodium dihydrate of EDTA individually per day by intraperitoneal injection. For groups D and E it was added to the chow at levels of 0.3 and 1.5% respectively.

Fox chow has a high content of calcium and iron. From the amounts eaten the daily intake of each rat was 140–210 mg of calcium and 3–5 mg of iron. From the diets which contained the EDTA compound they received 30–45 and 150–225 mg of it per day. In these diets the molar ratios of EDTA:Ca:Fe were 1:49:0.8 and 1:9.8:0.16.

One to four rats in each group were removed on the 56th day of the experiment because of respiratory infection. Otherwise all survived to its completion except one in group C and two in group E.

On the 110th day 13–15 rats from each of groups A, B, and C, and on the 111th day the same numbers from the other two groups, were placed individually in metabolism cages for the 24-hour collection of faeces and urine. After this, on the following day, all the rats in each group were killed. The liver and spleen were removed, and blood was taken from the heart. The right femur was removed from five animals in each group. The organs and bones which were removed were weighed. The calcium content of the faeces, urine, bone, and blood serum was determined by EDTA titration after ashing (2, 3). The iron content of liver (4), spleen (4), and serum (5) was determined. The serum from three to five animals was pooled for analysis, and two to four values were obtained from each group. Other values were obtained from individual animals. A few losses occurred from accidents during analysis.

Larger doses of EDTA disodium dihydrate were given to other rats from the same litters, as follows: Two groups of 9 animals each were fed respectively chow containing 3% and 5%, and 20 were fed chow containing 12%. Another 20 were given by intraperitoneal injection 6–40 mg per day each. The details of the dosages are given in Table I, where the deaths and survivals among these animals are recorded.

Results and Conclusions

The details of treatment and survival of the rats referred to in the last paragraph are shown in Table I. All of those animals which received EDTA in the diet at levels of 3–12% ate less than those in groups A–E in the longer experiment. They were also affected with diarrhoea, which varied in severity with the amount of EDTA in the diet. Those which received the intraperitoneal doses of 6–40 mg per day were somewhat lethargic and anorectic.

These observations indicated that the highest levels which were given intraperitoneally and in the diet for the period of 110–111 days in groups A–E, i.e., 3 mg per day and 1.5% respectively, were near the maxima which

TABLE I

The survival of rats given large doses of EDTA

Dosage				Survival	
Intraperitoneal EDTA	EDTA in the diet	Number of rats	Deaths	Number of rats	Period of treatment
	3%	9	1 on 32nd day	8	53 days
	5%	9	2 on 11th day 2 on 25th day	5	53 days
	12%	20	20 on 1st day	0	1 day
6 mg/day for 11 days 10 mg/day thereafter		5	3 on 25th day 1 on 39th day	1	52 days
10 mg/day for 11 days 20 mg/day thereafter		5	1 on 32nd day	4	52 days
15 mg/day for 11 days 30 mg/day thereafter		5	1 on 4th day 1 on 32nd day	3	52 days
20 mg/day for 11 days 40 mg/day thereafter		5	2 on 25th day 1 on 32nd day 1 on 39th day 1 on 46th day	0	46 days

can be tolerated by rats throughout an important segment of their most active period of growth.

The results of the experiment with groups A–E are shown in Fig. 1 and Table II.

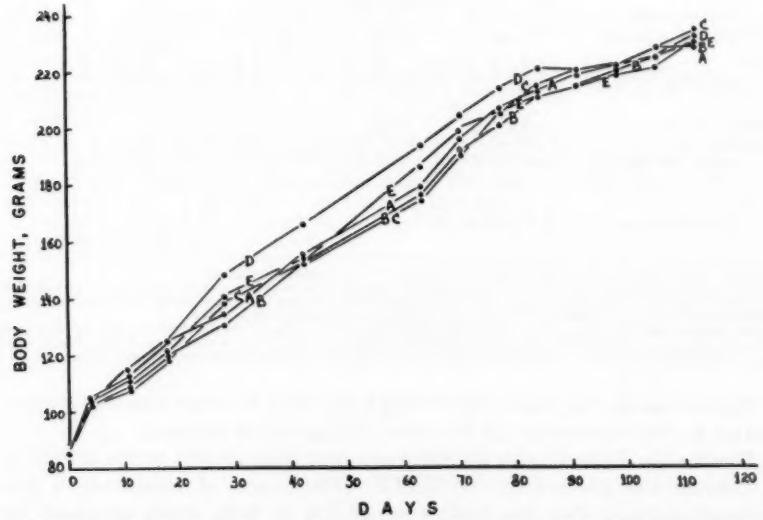


FIG. 1. The growth of rats receiving various amounts of the dihydrate of disodium EDTA. A, control; B and C, 0.5 and 3.0 mg per day intraperitoneally; D and E, 0.3 and 1.5% in the diet.

TABLE II

The utilization of calcium and iron by rats given EDTA (regimens A–E)
(Values given as ranges, averages, and standard deviations, with *n* values in parentheses)

	A	B	C	D	E
		Intraperitoneal EDTA		EDTA in the diet	
	(control)	0.5 mg/day	3 mg/day	0.3%	1.5%
		Calcium			
Feces	(11)	(12)	(11)	(12)	(11)
Total Ca, mg/day	173 – 465	165 – 343	159 – 453	220 – 433	168 – 365
	301 ± 74	247 ± 54	275 ± 90	304 ± 54	266 ± 63
Urine	(11)	(12)	(12)	(12)	(12)
Total Ca, mg/day	0.8 – 3.0	0.7 – 1.8	1.0 – 2.1	1.0 – 2.4	0.8 – 3.3
	1.6 ± 0.58	1.3 ± 0.35	1.6 ± 0.39	1.6 ± 0.49	1.7 ± 0.89
Serum	(4)	(4)	(3)	(3)	(2)
Ca, mg/100 ml	10.3 – 10.5	9.8 – 10.5	10.4 – 10.6	9.9 – 10.4	10.6 – 10.6
	10.4 ± 0.08	10.2 ± 0.42	10.5 ± 0.10	10.2 ± 0.23	10.6 ± 0
Bone	(5)	(5)	(5)	(5)	(5)
Ratio,	0.21 – 0.34	0.21 – 0.30	0.25 – 0.34	0.21 – 0.33	0.21 – 0.30
femur wt/body wt	0.27 ± 0.06	0.25 ± 0.04	0.31 ± 0.04	0.29 ± 0.05	0.26 ± 0.04
	(5)	(5)	(5)	(5)	(5)
Ca,	13.7 – 17.7	15.7 – 18.5	15.0 – 16.1	15.2 – 16.8	15.5 – 17.3
g/100 g femur	15.8 ± 1.8	16.6 ± 1.1	15.5 ± 0.4	16.1 ± 0.7	16.2 ± 0.7
		Iron			
Serum	(4)	(4)	(3)	(3)	(2)
Fe,	0.20 – 0.35	0.23 – 0.35	0.22 – 0.29	0.28 – 0.48	0.34 – 0.38
mg/100 ml	0.27 ± 0.02	0.26 ± 0.04	0.26 ± 0.03	0.37 ± 0.10	0.36 ± 0.02
				$p \ll 0.01$	$p \ll 0.01$
Liver	(19)	(18)	(18)	(16)	(15)
Ratio,	3.2 – 4.7	2.3 – 4.7	2.7 – 4.5	3.2 – 4.0	2.1 – 4.1
liver wt/body wt	3.7 ± 0.39	3.6 ± 0.52	3.4 ± 0.37	3.6 ± 0.22	3.4 ± 0.56
	(19)	(18)	(18)	(16)	(15)
Fe,	16.6 – 49.5	16.1 – 46.8	15.7 – 52.8	11.8 – 50.9	6.4 – 16.7
mg/100 g liver	29.2 ± 9.6	31.0 ± 10.1	32.3 ± 13.5	28.4 ± 13.6	10.2 ± 2.4
					$p \ll 0.01$
Spleen	(19)	(18)	(18)	(16)	(15)
Ratio,	0.08 – 0.25	0.10 – 0.19	0.13 – 0.25	0.14 – 0.24	0.12 – 0.24
spleen wt/body wt	0.17 ± 0.04	0.15 ± 0.03	0.18 ± 0.03	0.19 ± 0.03	0.17 ± 0.04
	(19)	(18)	(18)	(15)	(15)
Fe,	60.7 – 143.2	33.2 – 132.3	35.2 – 109.9	42.1 – 132.7	24.6 – 39.9
mg/100 g spleen	99.2 ± 34.6	86.1 ± 37.5	59.4 ± 17.1	77.1 ± 32.1	33.1 ± 5.3
			$p \ll 0.01$	$p \ll 0.01$	$p \ll 0.01$

NOTE: These results were obtained after 110–112 days on the regimens indicated.
Data on faeces and urine are from 11–12 rats in each group, those on femur from 5, and those on serum, liver, and spleen from 15 to 20 in each group. Individual values were obtained in all cases except for serum, which was pooled from three to five animals and two to four values were obtained in each group.
The p values which are given refer to the difference between the average for the group where they are shown and the control group A. These differences were statistically significant only where p values are shown. The p values were calculated as directed by Davenport and Ekas (6) as applied to differences between small samples.

Figure 1 shows that the levels of EDTA provided by either route of administration in this experiment did not affect the growth of the rats.

The data in Table II give no indication that bone growth or the utilization of calcium was affected by the EDTA. The ranges of values which were obtained indicate that the feeding of EDTA at both levels increased the concentration of iron in the blood serum, and that both the feeding of it and the injection of the higher intraperitoneal dose decreased the concentration of

iron in the spleen. The larger amount which was fed also decreased the concentration of iron in the liver. The size of the liver and of the spleen was not affected.

It is noteworthy that repeated doses of EDTA near the maximum which the growing rat can tolerate did not appear to affect the utilization of calcium by the animal. The very large dietary supply of calcium may have been responsible for this. When EDTA has been used in human medicine to reduce hypercalcaemia, the doses have been very large, and given intravenously. It does not reduce the serum calcium in normal individuals, but it has been shown to increase the excretion of calcium (see references in (1)).

It is important to consider the apparently preferential effect upon iron. The results suggest that if sufficient EDTA is given the iron stores are invaded. We have previously shown that the excretion of iron is increased (1). The high serum level is consistent with the intermediary role which that fraction would assume under those metabolic circumstances. The only discrepancy between the results of the present experiment and those of our experiment with Fe^{59} (1) is that the Fe^{59} content of the spleen increased when EDTA was fed; but this may have been a temporary state characteristic of an early stage during the ingestion of EDTA.

It is suggested that the greater affinity which EDTA has toward iron than toward calcium (7) is reflected in its effects in the animal body. It is therefore important that the state of iron nutrition should be considered if EDTA is ingested. Small amounts may be harmless, but the limit has not been defined.

References

1. B. A. Larsen, R. G. S. Bidwell, and W. W. Hawkins. Can. J. Biochem. and Physiol. **38**, 51 (1960).
2. A. A. Henly and R. A. Saunders. Analyst, **83**, 584 (1958).
3. H. M. C. Robinson and J. C. Rathbun. Can. J. Biochem. and Physiol. **37**, 225 (1959).
4. E. M. Scott. Arch. Biochem. Biophys. **6**, 27 (1945).
5. T. Peters, T. J. Givanniello, L. Apt, and J. F. Ross. J. Lab. Clin. Med. **48**, 280 (1956).
6. C. B. Davenport and M. P. Ekas. Statistical methods in biology, medicine and psychology. 4th ed. John Wiley & Sons, Inc., New York. 1936. p. 38.
7. Bersworth Chemical Co., Framingham, Mass. The versenes. 7th ed., Sect. I. 1954. p. 7.

THE EFFECT OF 2-AMINOETHYLISOTHIOURONIUM (AET) ON THE RESPIRATION OF MOUSE LIVER AND SPLEEN SLICES[1]

P. V. VITTORIO AND WILMA P. SPENCE

Abstract

The effect of AET injected in vivo on the respiration of liver and spleen slices from non-irradiated and X-irradiated mice was studied. The mice were killed 4 or 24 hours after the injection of a protective dose of AET and the livers and spleens were removed, sliced, and incubated for 3 hours in Krebs–Henseleit bicarbonate buffer containing uniformly labelled C^{14} glucose. In both non-irradiated and X-irradiated mice, initially, AET depressed the respiration of $C^{14}O_2$ from incubated liver slices but this depressing effect appeared to be overcome with time. Whole body X irradiation increased the total $C^{14}O_2$ respired by liver slices from mice injected with saline or AET prior to X irradiation and killed 4 or 24 hours after X irradiation. Whole body X irradiation decreased the total $C^{14}O_2$ respired by the spleen slices and the 24-hour samples showed the greatest decrease. In both the non-irradiated and X-irradiated mice AET did not appear to affect the total $C^{14}O_2$ respired by spleen slices.

Introduction

The discovery by Doherty and Burnett of the radioprotective activity of 2-aminoethylisothiouronium ·Br ·HBr (AET) in mice (1) has caused considerable interest in this compound. Although AET does not appear to be a very promising agent against radiation in man, knowledge regarding its mode of action in providing protection against radiation and the causes for survival could be very useful in the search to develop new and better protective agents against radiation. Using I^{131}, it has been shown by Vittorio *et al.* that AET lowers iodine uptake by the thyroids and by the serum proteins in both non-irradiated and X-irradiated rats (2). The same authors concluded that this might indicate that AET lowers the basal metabolic rate. The present report describes experiments using carbon-14 designed to determine the effect of AET injected in vivo on the in vitro respiration of liver and spleen slices. Both non-irradiated and X-irradiated mice which had been injected with a protective dose of AET were used.

Materials and Methods

Male 39-day-old mice weighing 25.0 ± 2.0 g were used. Aqueous solutions of AET were freshly prepared and neutralized with NaOH immediately prior to use. The required dose of AET (6.6 mg in 0.2 ml H_2O) was administered intraperitoneally. Food was withheld from all mice just prior to the injection of AET or saline, but they were permitted to drink water ad libitum.

Irradiation of Animals

Ten minutes after the injection of AET or saline the mice were irradiated with a total body dose of 625 r. X irradiation was carried out using a Mueller X-ray machine operating at 300 kvp, 10 ma, delivering an air dose of 28.5 r

[1]Manuscript received February 9, 1960.
Contribution from Radiobiology Department, Defence Research Board, Kingston, Ontario. Project No. D89-16-75-01.

per minute. The filtration employed was Al 0.127 g/cm² (one sheet) and Cu 0.888 g/cm² (two sheets). During irradiation, the mice were enclosed in a circular lucite cage which was rotated at 4 revolutions per minute. The non-irradiated control mice were also rotated in a similar lucite cage at the same rate.

Incubation Procedures

The mice were killed by a blow on the head, the livers and spleens were removed immediately, chilled, and sliced. Slices 0.5 mm thick were prepared with a McIlwain mechanical tissue slicer. The livers or spleens from six mice of the same group were sliced and pooled and 500 mg of liver or spleen slices were placed in the outer compartment of a 50-ml incubation flask which contained 35 μg of uniformly labelled C^{14} glucose (having a total activity of 70×10^5 counts/min) dissolved in 5.0 ml of Krebs–Henseleit bicarbonate buffer (3). The uniformly labelled glucose was prepared by the authors according to the method of Vittorio (4). Incubations and analyses were carried out in triplicate and each result represents the average value obtained (5). The flasks were gassed with a mixture of 95% O_2 and 5% CO_2. They were then capped with a self-sealing rubber stopper and incubated in a Dubnoff metabolic shaker (37.5° C) for 3 hours. At the end of the incubation period approximately 0.5 ml of 10% KOH was injected into the center well of the flask by means of a long needle attached to a hypodermic syringe. In a similar manner 1*N* HCl was added to the incubation medium (pH 3) to inactivate the tissue.

At least 30 minutes were allowed for CO_2 absorption. The contents of the center well were then transferred to a round-bottom flask and the CO_2 was released *in vacuo* by the addition of acid and trapped in dilute (0.05 *N*) NaOH. The CO_2 was precipitated with $BaCl_2$ as $BaCO_3$ and the total amount of CO_2 present was determined by titrating the excess NaOH with HCl. The $BaCO_3$ was washed with distilled H_2O and plated as a slurry on weighed aluminum trays. The samples which were prepared in triplicate were dried under infrared lamps and reweighed. They were then counted in a methane gas flow counter and the total activity of the respired carbon was calculated.

Results and Discussion

Liver and spleen slices were prepared as described from non-irradiated and X-irradiated mice injected with saline or a protective dose of AET. The mice were killed 4 or 24 hours after the injection of saline or AET. For convenience they will be referred to as the 4- or 24-hour sample. Table I shows the total activity in the $C^{14}O_2$ respired by each of the liver slice samples. In the non-irradiated 4-hour sample, the liver slices from the AET-injected mice respired about 20% less $C^{14}O_2$ than did the saline-injected controls, so that AET appeared to depress respiration. In the irradiated 4-hour samples, the liver slices from the AET-injected mice respired about 74% less $C^{14}O_2$ than the corresponding saline-injected mice. The saline- or AET-irradiated 4-hour samples both respired more $C^{14}O_2$ than did the corresponding non-irradiated controls. This provides further evidence that initially, X irradiation increases the respiration rate of liver slices (5).

TABLE I

The utilization of C^{14} glucose in respiration by 500 mg of liver slices from non-irradiated and X-irradiated mice incubated for 3 hours in Krebs–Henseleit bicarbonate buffer at 37.5° C

Compound injected I.P.	Time after X irradiation (hr)	Carbon-14 respired as $C^{14}O_2$	
		% of glucose* activity	% of control†
Non-irradiated			
Saline	4	3.1	100 ± 4.0
A.E.T.	4	2.5	80 ± 4.1
Irradiated			
Saline	4	6.5	209 ± 4.5
A.E.T.	4	4.2	135 ± 3.9
Non-irradiated			
Saline	24	5.5	177 ± 4.2
A.E.T.	24	5.4	174 ± 3.8
Irradiated			
Saline	24	5.8	187 ± 4.5
A.E.T.	24	9.6	309 ± 4.3

*All samples were incubated with the same amount of C^{14} glucose containing the same total activity.
†The saline, 4-hour, non-irradiated sample was arbitrarily given a value of 100% and the other samples were expressed as a percentage of this.

In the non-irradiated 24-hour samples the total carbon-14 respired as $C^{14}O_2$ by the liver slices from the saline- or AET-injected mice was almost the same so that the depressing effects of AET appear to have worn off with time. In the irradiated 24-hour samples, the AET sample respired much more $C^{14}O_2$ than the saline sample. A possible explanation for this may be that since AET initially depressed respiration and since X irradiation increased total $C^{14}O_2$ respired as shown by the X-irradiated saline sample, as the depres-

TABLE II

The utilization of C^{14} glucose in respiration by 500 mg of spleen slices from non-irradiated and X-irradiated mice incubated for 3 hours in Krebs–Henseleit bicarbonate buffer at 37.5° C

Compound injected I.P.	Time after X irradiation (hr)	Carbon-14 respired as $C^{14}O_2$	
		% of glucose * activity	% of control †
Non-irradiated			
Saline	4	27	100 ± 4.2
A.E.T.	4	30	111 ± 5.1
Irradiated			
Saline	4	24	87 ± 3.2
A.E.T.	4	23	86 ± 3.5
Non-irradiated			
Saline	24	31	114 ± 4.5
A.E.T.	24	29	107 ± 4.1
Irradiated			
Saline	24	18	67 ± 3.0
A.E.T.	24	18	67 ± 3.2

*All samples were incubated with the same concentration of C^{14} glucose containing the same total activity.
†The saline, 4-hour, non-irradiated sample was arbitrarily given a value of 100% and the other samples were expressed as a percentage of this.

sing effects of AET wear off the stimulating effects of X irradiation may still be present so that a much increased respiration rate is observed.

Table II shows the total carbon-14 respired as $C^{14}O_2$ by each of the mouse spleen slice samples. It is interesting to note the high amount of carbon-14 respired as $C^{14}O_2$ by the spleen slices in comparison to the liver slices. The saline non-irradiated spleen slices respired almost 10 times as much $C^{14}O_2$ as the saline non-irradiated liver slices. In all the spleen samples, non-irradiated or irradiated, 4 or 24 hours after injection, the AET had very little effect on the total $C^{14}O_2$ respired. However, in both the 4- and 24-hour samples the total $C^{14}O_2$ respired by the irradiated samples obtained from animals injected with saline or AET was lower than the corresponding non-irradiated samples. The greatest depression (about 40%) appears in the samples prepared 24 hours after X irradiation. Thus, whole body X irradiation appeared to lower the respiration of spleen slices.

Acknowledgment

The authors wish to thank M. J. Allen for her valuable technical assistance during this investigation.

References

1. D. G. Doherty and W. T. Burnett. Proc. Soc. Exptl. Biol. Med. **89**, 312 (1955).
2. P. V. Vittorio and M. J. Allen. Submitted for publication.
3. H. A. Krebs and K. Henseleit. Z. physiol. chem. Hoppe-Seyler's, **210**, 33 (1932).
4. P. V. Vittorio. Ph.D. Thesis, Queen's University, Kingston, Ont. 1953.
5. P. V. Vittorio, W. P. Spence, and M. J. Johnston. Can. J. Biochem. and Physiol. **37**, 787 (1959).

HYPOGLYCEMIC DRUGS AND THE DEXTRAN 'ANAPHYLACTOID' INFLAMMATION[1]

V. W. Adamkiewicz, R. J. Fitko,[2] and A. A. Fortier

Abstract

Tolbutamide (Orinase) (260 mg/kg s.c.), a hypoglycemic drug of the sulphonylurea class, sensitizes the normal rat (135–185 g) towards the 'anaphylactoid' inflammation induced by the administration of dextran (1 ml 6% w/v solution in saline s.c. per rat). However, the drug does not sensitize the alloxan diabetic rat towards this inflammation, nor does it alleviate the classical signs of alloxan diabetes. It is suggested that the sensitization of normal rats towards dextran by tolbutamide is mediated through insulin.

It was reported previously that insulin sensitizes the normal rat to the 'anaphylactoid' inflammation produced by the injection of dextran (1, 2, 3). Tolbutamide (Orinase), a hypoglycemic drug of the sulphonylurea type, simulates several actions of insulin. The present communication shows that it also sensitizes the normal rat towards the inflammatory response to dextran. This appears, however, to be mediated through insulin, because, in alloxan diabetic rats, in the absence of insulin, the drug loses its ability to sensitize.

Methods

The rats used were Sprague–Dawley males, 135–185 g in body weight, maintained on a diet of Purina cubes and water.

Diabetes was produced by injecting into the jugular vein of normal rats, anesthetized with ether, 8.65 mg of alloxan monohydrate (Eastman Organic Chemicals, roughly 50 mg per kg of body weight) dissolved in 0.25 ml of a 0.25 *M*, ice-cold citrate–phosphate buffer at pH 4. This alloxan solution was injected within the 60 minutes after preparation (4). The development of diabetes was checked as follows. For 7 days before the injection of alloxan, all the rats were weighed daily, the amount of water they consumed was measured in their drinking bottles, and their glucosuria was estimated by means of the Clinitest reagent tablets (Ames Co.) (5). On the seventh day, alloxan was injected, and for 5 days afterwards, until the 12th day of the experiment (Fig. 3), the daily loss of body weight and the increase in water consumption and glucosuria were determined. Only rats showing a decreasing or stationary body weight, a water consumption of at least 60 ml per rat per day, and a glucosuria of at least 1.00 g% were considered to be diabetic. As qualified by these conditions, 85% of rats injected with alloxan were diabetic on the 12th day. On that day, some were treated with insulin, some with tolbutamide, some were left untreated. All were injected intraperitoneally with dextran to study its 'anaphylactoid' effect. From the 12th to the 25th day, the daily treatment with insulin or tolbutamide was continued and the signs of diabetes were evaluated as before.

[1]Manuscript received February 11, 1960.
Contribution from the Laboratoire de Physiologie, Université de Montréal, Montreal, Que.
[2]Fellow of the Rockefeller Foundation. Present address: Department of Physio-pathology, Faculty of Veterinary Medicine, Main Agricultural College, Warsaw, Poland.

Dextran, a 6% w/v solution in physiological saline (Abbott) was always injected in the dose of 1 ml (60 mg) per rat. In the first experiment, this was injected subcutaneously on the back, and, in the second, intraperitoneally. Dextran administered subcutaneously on the back rarely induces an 'anaphylactoid' inflammation. The subcutaneous tissue there is not a site of predilection for this reaction (6). But, an 'anaphylactoid' inflammation is induced regularly if a sensitizing dose of insulin is administered as well (1). On the other hand, dextran injected intraperitoneally regularly induces an 'anaphylactoid' inflammation in normal rats. However, diabetic rats will not react even to intraperitoneal dextran unless they are treated with insulin (5). Two hypotheses have been suggested to explain these phenomena (7, 8).

In the first experiment, tolbutamide sodium (Horner) and compound F.W.H. 116 sodium (methoxy-benzenesulphamido-5-tertbutyl,1,3,4-thiadiazole; Horner) were injected subcutaneously in the dose of 40 mg per rat (roughly 260 mg per kg), dissolved in 1 ml of physiological saline. In the second experiment, only the tolbutamide was used and in the third experiment, the tolbutamide injections were repeated daily.

Insulin-Zn ("Insulin-Toronto," Connaught) was administered at a dose of 0.16 mg (4 units) per rat subcutaneously (0.1 ml commercial preparation) during the first and second experiment. In the third experiment, Protamine Zinc Insulin (Connaught) was administered by daily subcutaneous injections in the dose of 2 units per rat during the first week, and 4 units during the second.

The intensity of the general 'anaphylactoid' inflammation was estimated in milliliters by measuring the changes of the volume of the left hind paw, hourly, during 5 to 6 hours after the injection of dextran (9). Depending upon the experimental conditions, either all the rats became inflamed within 3 hours after injection of dextran, or none became inflamed.

Results

1. *Sensitization by Hypoglycemic Drugs to the Dextran 'Anaphylactoid' Inflammation in Normal Rats*

Four groups of 10 normal rats were used. All the animals were injected with dextran subcutaneously. Group I control rats had no other treatment. Group II received in addition insulin; group III, tolbutamide; and group IV, compound F.W.H. 116.

The degree of inflammation was negligible in the controls. The insulin-treated and tolbutamide-treated rats reacted by an intense 'anaphylactoid' inflammation. The volume of their paws before inflammation was about 0.90 ml. During inflammation, it increased to 1.40–1.50 ml (Fig. 1). The rats treated with compound F.W.H. 116 also reacted to dextran, but to a lesser degree than the insulin or tolbutamide groups. This compound was about half as active as tolbutamide and was eliminated from further experiments.

2. *Tolbutamide and the Dextran 'Anaphylactoid' Inflammation in Diabetic Rats*

Three groups, each of 20 diabetic rats, were used. All the animals were injected with dextran intraperitoneally. Group I control rats had no other treatment; group II received insulin; and group III, tolbutamide.

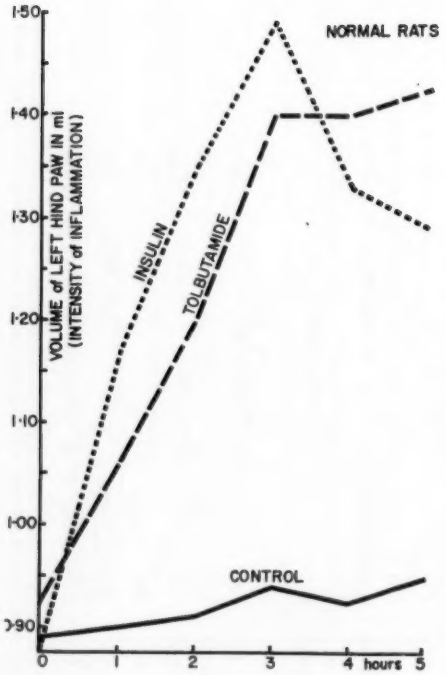


FIG. 1

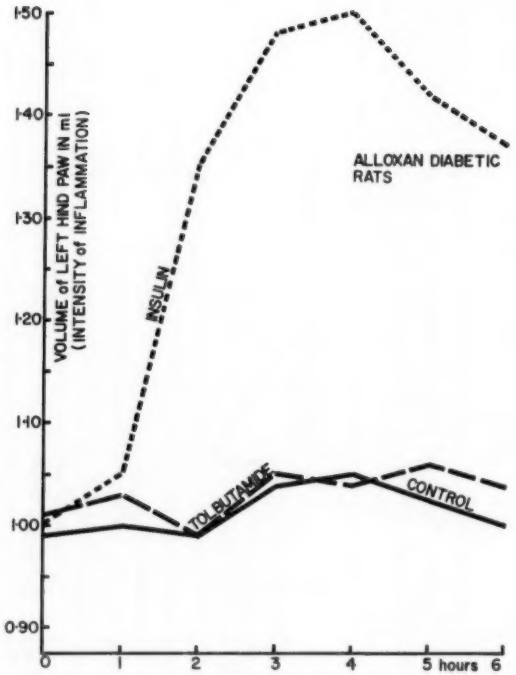


FIG. 2

FIG. 1. Sensitization by tolbutamide to the dextran 'anaphylactoid' inflammation in normal rats. Three groups of normal rats were injected with dextran into a site of non-predilection. Only rats sensitized with insulin or tolbutamide became inflamed.

FIG. 2. Tolbutamide and the dextran 'anaphylactoid' inflammation in diabetic rats. Three groups of diabetic rats were injected with dextran into a site of predilection. Only the insulin-treated animals reacted by an 'anaphylactoid' inflammation. (Refer also to Fig. 3.)

Only the insulin-treated rats reacted by an 'anaphylactoid' inflammation (Fig. 2). All these rats then were used again for experiment 3.

3. *Effects on Alloxan Diabetic Rats of Chronic Treatment with Insulin or Tolbutamide*

The three groups of diabetic rats from the previous experiment were treated for 13 days as follows. The first group remained as controls. The second received daily injections of insulin; and the third, daily injections of tolbutamide.

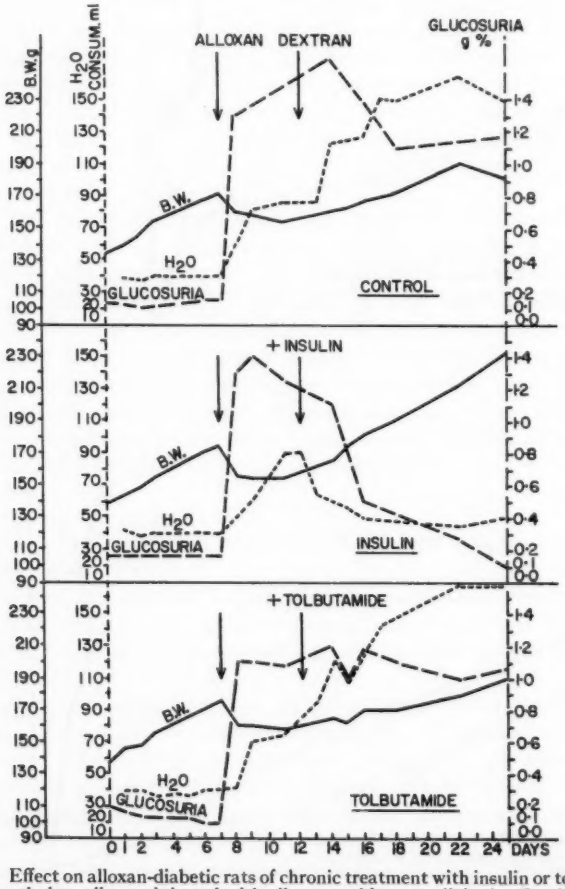


FIG. 3. Effect on alloxan-diabetic rats of chronic treatment with insulin or tolbutamide. On the seventh day, all were injected with alloxan and became diabetic. On the 12th day, all the rats were injected intraperitoneally with dextran and, in addition, group II received insulin and group III, tolbutamide. (The result of this treatment is shown in Fig. 2.) During the subsequent 13 days, group I rats remained as controls, group II received daily insulin, and group III daily tolbutamide. The signs of diabetes disappeared only in the insulin group.

The control animals continued to display signs of acute diabetes. Their average daily body weight increase was 0.8 g per rat (the normal value is 3–6 g). Their water consumption was high, about 160 ml per rat per day (normal: 25–40 ml). Their glucosuria was increased: 1.1 g% (normal: 0–0.25 g%). The insulin-treated rats lost the signs of diabetes completely. Their body weight increased by an average of 5.5 g per rat per day. Water consumption was about 50 ml, and glucosuria disappeared. In tolbutamide-treated animals, the signs of diabetes remained as intense as in the controls, but their physical condition was considerably worse than that of the controls (Fig. 3).

Discussion

Two mechanisms may be postulated to account for the sensitizing effect of hypoglycemic drugs on the 'anaphylactoid' inflammatory response to dextran, in normal rats. In the first instance, the drugs would have their own specific sensitization mechanism, independent of insulin. In the second, they would act through insulin; either by discharging more insulin into the animal, or by potentiating the action of insulin already existing in the animal.

Alloxan-diabetic rats do not undergo the dextran 'anaphylactoid' inflammation, but the inflammation may be restored by administration of insulin. Therefore, this inflammation depends on the insulin content of the rat.

Tolbutamide (and other hypoglycemic drugs) sensitizes to dextran the normal rat which contains a normal amount of insulin. But it does not restore the inflammatory response in diabetic rats which do not contain insulin; in the absence of insulin, the drug loses its sensitizing property. Therefore, the sensitizing property of tolbutamide is dependent on the insulin content of the rat. This conclusion is further supported by the observation that tolbutamide does not alleviate the other (classical) signs of diabetes in the rat, such as increased water consumption, glucosuria and diminished body growth.

It may be pointed out also that a sensitization to the inflammatory response to dextran in normal rats is induced by 0.16 mg (4 units) of insulin-Zn per animal. To obtain the same effect with tolbutamide, 40 mg per rat are required. This quantitative difference indicates that, in the present instance, roughly one molecule of insulin does the work of 11,000 molecules of tolbutamide.

Acknowledgment

This study was supported by research grant No. MA 640 from the National Research Council of Canada and research grant No. A-2260 from the U.S. Department of Health, Education and Welfare.

References

1. V. W. Adamkiewicz and Y. Langlois. Can. J. Biochem. and Physiol. **35**, 251 (1957).
2. L. M. Adamkiewicz and V. W. Adamkiewicz. J. Physiol. (London), **145**, 667 (1959).
3. A. Goth, W. L. Nash, M. Naggler, and J. Holman. Am. J. Physiol. **191**, 25 (1957).
4. S. J. Klebanoff and A. L. Greenbaum. J. Endocrinol. **11**, 314 (1954).
5. V. W. Adamkiewicz and L. M. Adamkiewicz. Am. J. Physiol. **197**, 377 (1959).
6. H. Selye. J. Allergy, **25**, 97 (1954).
7. V. W. Adamkiewicz and L. M. Adamkiewicz. Am. J. Physiol. **198**, 51 (1960).
8. A. Goth. Am. J. Physiol. **197**, 1056 (1959).
9. V. W. Adamkiewicz, W. B. Rice, and J. D. McColl. Can. J. Biochem. and Physiol. **33**, 332 (1955).

THE APPARENT EXTRACELLULAR SPACE OF MAMMALIAN SKELETAL MUSCLE

A COMPARISON OF THE INULIN SPACE IN NORMAL AND DYSTROPHIC MOUSE TISSUES[1]

L. H. BURR AND H. MCLENNAN

Abstract

The apparent extracellular volumes of the muscles from young normal and dystrophic mice have been estimated, using inulin dilution techniques. The inulin spaces were measured in the muscle both following injection of inulin in vivo and after soaking of excised tissues in a solution containing inulin. Comparisons were made between muscles of different size from the same animal as well as from different animals whose age, and consequently muscle size, varied. In all cases it has been found that the inulin space decreases with increasing muscle size. Similar results have been obtained by others with toad sartorii. The inulin space in muscles from dystrophic mice is larger than that of comparable normal tissues, and the dependence on muscle size, although similar to normal, is more pronounced. The results suggest that the dystrophic cells are permeable to inulin, and the question that some small permeability may be present also in normal tissue is considered.

Introduction

The polysaccharide inulin has long been regarded as a substance which is unable to penetrate cellular membranes, and has in consequence been used to estimate the volume of the extracellular fluid in a number of tissues. Methods depending on its equilibration in the extracellular fluid either after systemic injection of the substance or after in vitro incubation of the tissue in an appropriate medium have indicated that fairly constant and reproducible values can be obtained. In the case of muscle the inulin space has been found to be smaller than the corresponding chloride space, consistent with the fact that although chloride is predominantly an extracellular ion, it is present in small concentration within the cells. Thus, in the rat, Wilde (1) reported 17% for the chloride space and 13.5% for the inulin space of muscle. Other workers have reported similar values for the inulin space of rat muscle (Creese, D'Silva, and Hashish (2); McLennan (3)).

In the present study the apparent extracellular volumes of skeletal muscles from dystrophic and normal mice were compared, and in the light of differences found, a study of muscles of different sizes (either different muscles from the same animal, or comparable muscles from animals of different size) has also been made. It has been found that there is a negative correlation between inulin space and muscle size, and that, in addition, the dystrophic muscle cells appear to be penetrated by the inulin.

Methods

The animals used were from a pure inbred Bar Harbour 129 strain of mice obtained from the colony presently maintained at the University of British

[1]Manuscript received March 28, 1960.
Contribution from Department of Physiology, University of British Columbia, Vancouver 8, B.C.

Columbia, with the addition of a few Swiss albino animals. The 129 strain develops a disorder of muscle, transmitted as an autosomal recessive trait, which closely resembles human progressive muscular dystrophy, both genetically and histologically (Michelson, Russell, and Harman (4)). Litter mates which did not develop the condition provided normal animals.

Experiments were carried out in vitro with m. gastrocnemius. Excised muscles were weighed and incubated at 33° C for a standard period of 4 hours in Locke's solution equilibrated with 95% O_2 – 5% CO_2, and containing 1% (w/v) inulin. McLennan (3) had shown that for the much larger muscles of the rat this time was sufficient for the complete equilibration of the extracellular space with the bathing fluid. In that study it was shown that the uptake of inulin by muscles weighing about 100 mg was essentially complete in $2\frac{1}{2}$ hours, and that the measured inulin space was unchanged if the incubation time was extended to 6 hours. At the end of incubation the muscles were blotted and weighed again to determine the degree of swelling which had occurred, and were then ground with sand, suspended in 3 ml of water, and 1 ml of 12% trichloroacetic acid was added. The mixture was centrifuged and aliquots of the supernatant solution assayed for inulin.

For estimation of the uptake of inulin in vivo bilateral ligation of the renal artery and vein was carried out under ether anaesthesia, and 0.3 ml of 25% (w/v) inulin solution injected under the skin of the back. After a variable period of time ($\frac{1}{2}$ to $3\frac{1}{2}$ hours) the animals were killed by exsanguination from the heart and the desired muscles (gastrocnemius or peroneus longus) were excised, weighed, and treated as above. The blood was placed in a tube containing a small known amount of heparin solution, centrifuged, and 0.1-ml aliquots of the plasma were treated with TCA and assayed for inulin.

Determination of inulin was carried out by the method of Hubbard and Loomis (5). All inulin spaces are expressed, after correction in the case of the in vitro experiments, for the amount of inulin contained in the water of swelling (Pappius and Elliott (6)), as

$$\frac{\text{mg inulin per g fresh weight of muscle} \times 100}{\text{mg inulin per ml plasma or soaking solution}} \%.$$

Experiments were also carried out in vitro to determine the thiocyanate space of the muscles. They were incubated in Locke's solution containing 48 meq/liter CNS^- (replacing chloride) for 1 hour, after which the muscles were treated as above and the thiocyanate determined by the method of Crandall and Anderson (7).

Results

The estimated inulin spaces determined in the m. gastrocnemii of normal mice after soaking of the tissues in an inulin-containing solution are set forth in Fig. 1. The smallest muscles (ca. 10 mg) were obtained from animals 10 to 12 days old who had body weights of 6 to 8 g, and larger muscles from progressively older animals. The oldest animals used were about 6 weeks old and weighed about 20 g.

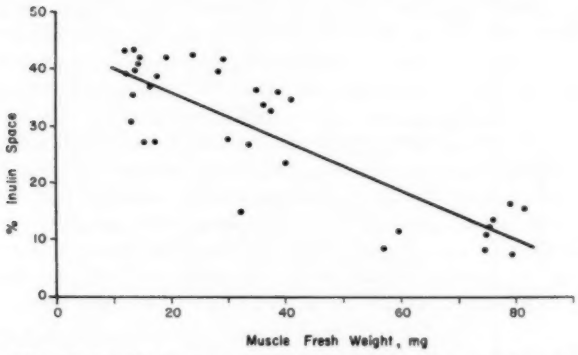


FIG. 1. The relationship between muscle weight and inulin space in gastrocnemii of normal 129 strain mice. In vitro method. In this and succeeding figures each point represents the inulin space of an individual muscle.

It is evident that there is a relationship between the weight of the muscle and the inulin space. The calculated regression line is $y = 44.7 - 0.430x$ (S.D. ± 6.7; $n = 34$), and the correlation coefficient is highly significant ($r = -0.847$, $P < 0.001$).

When a further series of determinations was made after parenteral administration of inulin a similar relationship between muscle size and inulin space was observed (Fig. 2). However, in this case it was possible also to determine the inulin contained in a naturally small muscle (peroneus longus) in addition to that in the much larger gastrocnemius; thus each animal could provide a pair of large and a pair of small muscles for comparison. The calculated relationship between inulin space and muscle weight for this series was $y = 54.0 - 0.472x$ (S.D. ± 13.5, $n = 23$). Again the correlation coefficient was significant ($r = -0.629$; $P \doteqdot 0.001$). There is no significant difference between this line and that calculated for the result shown in Fig. 1.

In the light of this, and of the relatively small numbers of dystrophic animals available to us, the results obtained after soaking gastrocnemii and following injection of inulin into these animals have been pooled, and are shown in Fig. 3. The peronei longi of these mice were too small for estimation of their inulin content following injection. The age of the animals used was $3\frac{1}{2}$ to 4 weeks, at which time their body weights were 8 to 12 g. No attempt was made to assess the degree of dystrophic change involved, but all animals evidently had severe wasting of their hind limbs. Electrical stimulation revealed, however, that some residual contractility of the gastrocnemii remained in all cases.

Again a relationship between muscle weight and inulin space was evident, the calculated regression line being $y = 94.6 - 1.65x$ (S.D. ± 11.8; $n = 17$) ($r = -0.804$, $P < 0.001$). The smallest muscles had apparent extracellular spaces in excess of 90%. The largest dystrophic muscle used (ca. 40 mg) was from an animal in which the disease was progressing much more slowly than usual.

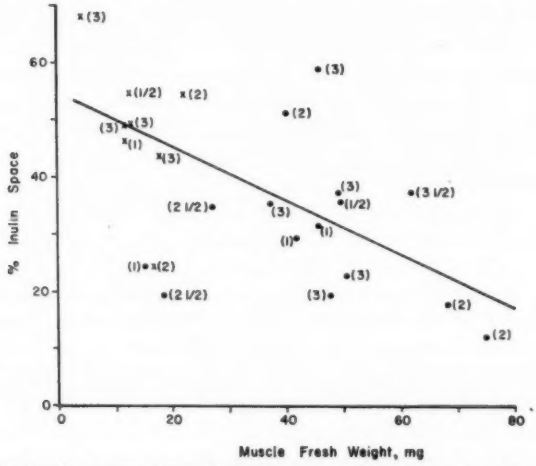


FIG. 2. The inulin space of muscles from normal 129 strain mice, determined after injection of the substance. ● = Gastrocnemii. × = Peronei longi. The figures in parentheses beside each point show the number of hours between injection and sacrifice of the animals.

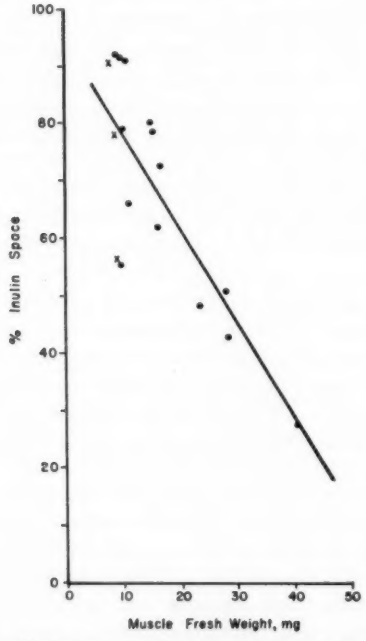


FIG. 3. The relationship between inulin space and muscle weight in gastrocnemii of dystrophic mice. ● In vitro method. × Animals injected with inulin 3 hours before sacrifice.

While these experiments were in progress, Tasker, Simon, Johnstone, Shankly, and Shaw (8) published results obtained on the toad *Bufo marinus*, which showed that toad sartorii also exhibit a negative correlation between inulin space and muscle size. However, we are aware of no description of the dependence of the inulin space of mammalian skeletal muscle on growth such as has been described here, and therefore we have made a few observations on Swiss albino mice as a species in which inherited muscular changes of the type shown by the 129 strain are unknown. It seemed wise to eliminate the possibility that the observed effect was peculiar to the latter strain; that it perhaps reflected in the 'normals' a subclinical change which was more pronounced in the 'dystrophics'. The calculated relationship holding in this case is $y = 39.8 - 0.249x$ (S.D. ± 5.7; $n = 23$) ($r = -0.754$, $P < 0.001$). This line is not significantly different from those of Figs. 1 and 2.

Some experiments have also been performed in which muscles from normal animals of the 129 strain were incubated in Locke's solution containing thiocyanate, and the proportion of the muscle occupied by this ion estimated. The calculated regression line is

$$y = 35.7 - 0.196x \text{ (S.D. } \pm 5.9;\ n = 23) \ (r = -0.544,\ P \doteqdot 0.01).*$$

In this instance the slope of the line is not significantly different from zero, nor from those of Figs. 1 and 2, although there is an observable trend for the thiocyanate space to decrease with increasing muscle weight.

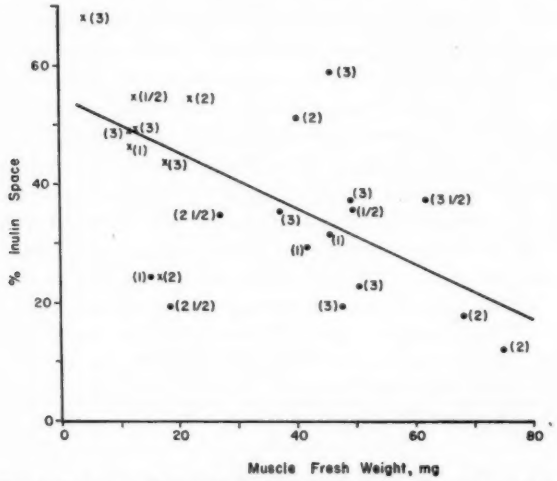
Discussion

Previous workers have used inulin for two main purposes: (*a*) either to measure the total volume of extracellular space in the whole animal (e.g. Kruhoffer (9)), or (*b*) to measure the volume in comparable samples of tissue from many animals (e.g. Creese *et al.* (2)). The present study shows that there is a progressive decrease in inulin space in mammalian skeletal muscle with increasing muscle size, and the trend is also evident if thiocyanate is used in place of inulin. This effect is similar to that recently reported by Tasker *et al.* (8) for the inulin space of toad muscles.

The effect would seem to be related to the ontogenesis of the muscle, and not to be a peculiarity of the strain of mice used or of the experimental method. Thus similar results were obtained for the Swiss albino mice as with the 129 strain, and similar results were obtained irrespective of whether equilibration of the inulin in the extracellular space took place in vivo or in vitro. Comparing gastrocnemii from normal animals of increasing age then, the relative volume of cytoplasm increases. Since the number of muscle cells is believed to be fixed at birth (10), and since the proportion of the total volume which is extracellular decreases, it follows that during growth the size of each cell increases faster than does the total volume of the muscle.

In comparing gastrocnemii and peronei longi from the same animal, the general observation is again apparent that small muscles have relatively

*The values for the apparent extracellular space have not been corrected for the quantity of thiocyanate likely to have been taken up by the cells (Pappius and Elliott (6)). The values for the thiocyanate space will be ca. 4% high from this cause.



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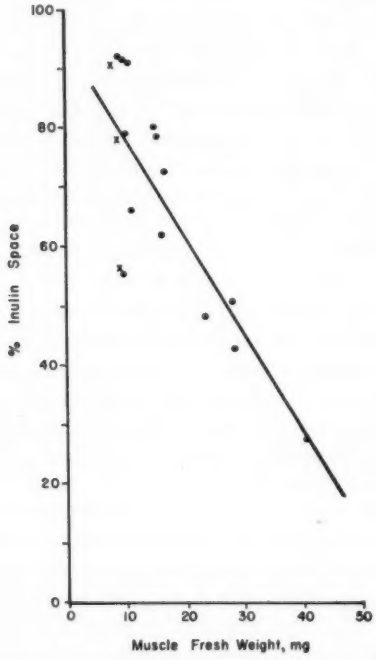

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larger apparent extracellular spaces than do large ones. In this instance, however, developmental effects cannot be involved, and the only likely explanation is that the individual sizes of the cells relative to that of the whole muscle is different for the two muscle types. We have no histological data to correlate with this.

The linear relation holding between muscle weight and inulin space shows a much steeper slope for dystrophic muscles than does that for the gastrocnemii of normal animals, such that the smallest dystrophic muscles tested had apparent extracellular spaces of ca. 90%. The dystrophic animals used were all about 4 weeks old and their gastrocnemii ranged in weight (with one exception) from ca. 10 to 30 mg, with corresponding spaces of ca. 90 to 40%. Normal animals of the same age had muscles weighing about 60 mg (20 to 25% inulin space); normal muscles in the same weight range as the dystrophics had spaces of ca. 40–35%. The dystrophic tissues then always had a larger inulin space than normal muscles either of the same age or of the same muscle weight, and for the latter comparison the discrepancy was greater the smaller the muscles.

The effect is presumably related to the degree of dystrophic change which had occurred in the muscle. As mentioned earlier the ages of the dystrophic animals fell in a narrow range and the differences in muscle weight largely reflect differences in the clinical state of the animals. Inulin spaces in excess of 90% would seem to indicate few functioning muscle cells, if the inulin were entirely confined to the extracellular compartment; yet as mentioned above visible twitches to electrical stimulation of the muscles could always be observed, and resting and action potentials could be recorded from them without difficulty (Burr and McLennan, unpublished observations). It seems reasonable to conclude, then, that in the dystrophic muscle inulin is in fact not so confined, and that a proportion of the amount found in the tissue after equilibration either in vivo or in vitro is contained within the cytoplasm. Such a change in the permeability of the cellular membrane is suggested also by the results of Zierler (11), who has shown that an increased leakage of the enzyme aldolase exists in muscle from dystrophic mice.

The question then arises to what extent a permeability of the cellular membrane to inulin in the normal tissues may occur. Zierler has provided evidence that there is a leak of aldolase also from normal mouse (11) and rat (12) muscle. The fact that the leakage of aldolase is increased in dystrophic tissue, and that there is reason to suspect an inward movement of inulin in this tissue also, suggests that perhaps in normal muscles some inulin does enter the cells and that the measured inulin space is not an exact index of the true extracellular space of a tissue.

Acknowledgments

We are indebted to Dr. J. R. Miller, Department of Neurological Research, University of British Columbia, for providing normal and dystrophic mice of the 129 strain. This work was supported by a generous grant from the Muscular Dystrophy Association of Canada to H. McL.

References

1. W. S. WILDE. Am. J. Physiol. **143**, 666 (1945).
2. R. CREESE, J. L. D'SILVA, and S. E. E. HASHISH. J. Physiol. **127**, 525 (1955).
3. H. McLENNAN. Biochim. et Biophys. Acta, **21**, 472 (1956).
4. A. M. MICHELSON, E. S. RUSSELL, and P. J. HARMAN. Proc. Natl. Acad. Sci. **41**, 1079 (1955).
5. R. S. HUBBARD and T. A. LOOMIS. J. Biol. Chem. **145**, 641 (1942).
6. H. M. PAPPIUS and K. A. C. ELLIOTT. Can. J. Biochem. and Physiol. **34**, 1007 (1956).
7. L. A. CRANDALL, JR. and M. X. ANDERSON. Am. J. Digest. Diseases Nutrition, **1**, 126 (1934).
8. P. TASKER, S. E. SIMON, B. M. JOHNSTONE, K. H. SHANKLY, and F. H. SHAW. J. Gen. Physiol. **43**, 39 (1959).
9. P. KRUHOFFER. Acta Physiol. Scand. **11**, 16 (1946).
10. A. W. HAM. Histology. 3rd ed. J. B. Lippincott Co., Philadelphia. 1950. p. 287.
11. K. L. ZIERLER. Bull. Johns Hopkins Hosp. **102**, 17 (1958).
12. K. L. ZIERLER. Am. J. Physiol. **190**, 201 (1957).

AMINOACIDURIA IN RATS AFTER TREATMENT WITH THE ETHYL ESTER OF GAMMA-BUTYROBETAINE[1]

ESAU A. HOSEIN, MARGARET SMART, AND KATHLEEN HAWKINS

Abstract

Previous workers have shown that the urine of patients with muscular dystrophy contains excessive amounts of amino acids. Using paper chromatographic analysis, these workers found that the amino acid methylhistidine is among those excreted in large amounts. Rats do not normally excrete methylhistidine, but in our experiments it was found that intramuscular injection of the ethyl ester of gamma-butyrobetaine into rats caused both the excretion of methylhistidine and an excessive aminoaciduria, presumably through the breakdown of muscle proteins.

Introduction

Ames and Risley (1), and later Blahd, Bloom, and Drell (2), using paper chromatographic methods of analysis, showed that the urine of patients with progressive muscular dystrophy contained many amino acids in large amounts. Methylhistidine was found among these amino acids. Rats do not normally excrete methylhistidine, although it is present in the urine of some carnivorous animals (3). The appearance of methylhistidine in the urine presumably is the result of anserine hydrolysis. Anserine is a dipeptide found in muscle (4) and brain (5) and recently Severin *et al.* (6) have provided evidence that this dipeptide functions in the body as a cofactor for creatine phosphate synthesis. Chemical analysis of muscle from muscular dystrophic patients has shown that the creatine phosphate content is lower than that found in normal tissue (7). The dipeptidase anserinase has been found to be present in muscle tissue (8) and Weinstock *et al.* (9) have shown that there is increased dipeptidase activity in the muscles of dystrophic patients. The aminoaciduria in patients with muscular dystrophy could be due to a rapid catabolism of muscle proteins or to inhibition of protein synthesis. Chemical analyses of biopsy specimens from dystrophic patients have shown that there is an increase in both the fat and collagen content of these tissues as the muscle mass decreases (10, 11). The adenosine triphosphate (ATP) content of muscle from dystrophic patients also is low (7) and so are many glycolytic enzymes such as aldolase, phosphorylase, and phosphohexoisomerase (12, 13). This loss of essential enzymes could probably explain the low ATP in these tissues, since the activity of the energy-yielding systems is diminished.

Experiments by Hosein and his associates (in preparation) have shown that the intramuscular injection of the ethyl ester of gamma-butyrobetaine (gBBE) into rats caused, among other effects, a loss of muscle potassium, a decrease of certain glycolytic enzymes, diminished creatine phosphate and ATP in muscle and in urine (14), an increased excretion of creatine and pentose with a decreased excretion of creatinine. These effects produced by gBBE in the rat are identical with some of the metabolic abnormalities found in muscular dystrophy and

[1]Manuscript received in original form December 28, 1959, and, as revised, March 30, 1960. Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

certain other muscle diseases. The results from our experiments indicated that the intramuscular injection of gBBE into rats produced a serious derangement of normal muscle metabolism. Thus it was important to investigate whether other metabolites of interest to muscle disease might be eliminated in the urine of rats treated with gBBE.

Previous work by Hosein (15) has shown that gamma-butyrobetaine (gBB) occurs in the body as an ester, namely gBB CoA. The physiological properties of this substance appear to be similar to that of acetylcholine, as tested on various assay preparations. In these studies, we have used the synthetic gBBE instead of the gBB CoA, as the latter is only available in tiny quantities and in addition, from the results of tests performed with these two substances, their pharmacological action appears to be identical. In the experiments described in this paper, it was found that the intramuscular injection of gBBE into rats caused intense aminoaciduria and the excretion of methylhistidine.

Materials

gBBE was prepared synthetically by the condensation of the ethyl ester of gamma-bromobutyric acid with anhydrous trimethylamine in a sealed tube at room temperature. The purity of the product was confirmed by combustion analysis and by paper chromatography in the butanol–water system (R_f 0.21).

Methylhistidine was obtained from the California Corporation for Biochemical Research.

In all of these experiments normal adult albino rats, of either sex but of uniform weight, were used throughout.

Experimental Procedures

The rats used in these experiments were kept on a standard diet for several days before treatment with either saline or gBBE. During the control period, which lasted 1 week, 0.5 ml of saline was injected intramuscularly into each hind leg of the rat, three times daily. During the experimental period, which was of similar duration, 5 mg gBBE was dissolved in the 0.5 ml saline solution. After each injection with the gBBE, the animal was paralyzed for about 20–30 minutes, during which time it salivated profusely and secreted "bloody tears". Twenty-four-hour urine samples were collected daily before and after gBBE treatment and each sample was measured, centrifuged, decolorized with norite, and concentrated under reduced pressure at 40° C to a volume of 0.5 ml. In later experiments, rats were injected intramuscularly with 50 μg gBBE in 0.5 ml saline in each hind leg once daily for a period of 4 weeks. A single 24-hour urine sample was collected at the end of each week of treatment and this was processed as described above.

Paper chromatographic analysis was carried out on the concentrated samples of urine in the following manner. The samples, 0.1 ml, were spread on the starting line of large strips of Whatman No. 1 filter paper 6 in. wide and 18 in. long. After the strips were dried, the chromatogram was developed in a descending manner with propanol–water (70–30) mixture for 20 hours at room temperature. The papers were then dried in air. A marker strip, 1 in. wide,

was cut along the long axis of the paper and it was sprayed with a 1% solution of ninhydrin in ethanol to give an indication as to whether there was any ninhydrin positive material in the particular zone of the paper in which we were interested. The untreated portion of the chromatogram was then cut at the zone between R_f 0.28–0.32 and this was extracted with 20 ml of a methanol–water (50–50) mixture by shaking for 2 hours. The eluate was reduced to a volume of 0.1 ml at 40° C and this was applied as a single spot for the second dimensional development in phenol–water (3–1). Again the chromatogram was sprayed with ninhydrin after all the phenol had been removed by air-drying the paper for 24 hours at room temperature. Authentic samples of methylhistidine were used as standards in both chromatogram systems.

Results

(A) Methylhistidine in the Urine of Rats Treated with gBBE

In these studies, the rat was selected as the experimental animal since it does not normally excrete methylhistidine in the urine. Accordingly, in the first series of experiments, 5-mg doses of gBBE were administered to animals in order to determine whether this substance could produce this metabolic abnormality within a reasonably short period of time. In later experiments, the dosage of gBBE was reduced to 50 μg per injection and the period of administration was extended to 4 weeks, to determine whether this small quantity of material could produce the effects observed when larger dosages were used.

The results of one of several experiments when 5-mg dosages of gBBE were used are shown in Fig. 1. In the propanol–water system methylhistidine has an R_f of 0.30, while in the phenol–water system the R_f is 0.80. Material with the same R_f values as methylhistidine is evidently present in the sample of urine derived from rats treated with gBBE, since the material obtained in these chromatograms was originally extracted from the band with R_f 0.28–0.32 in the propanol–water system. No such material was found in the urine of saline-treated or untreated control animals. On the basis of the two-dimensional chromatographic analysis, it appears that the material detected in the urine of rats treated with gBBE most probably is methylhistidine.

From these experiments it was found that when the larger dosages of gBBE were used, the amino acid was excreted in the urine much earlier than with smaller amounts of the drug. In the former experiments, methylhistidine was found in the urine in small quantities after the third day of treatment and the amount increased daily. With the smaller amounts of gBBE, no methylhistidine was observed in the urine until the end of the 3rd week of treatment. At that time, the amino acid could just be detected. It was felt that, had the injections been continued for a much longer period of time, say several months, the urinary excretion of this amino acid would undoubtedly have increased, since it was observed that at the end of the 4th week of treatment, the excretion of methylhistidine could be more readily detected on the chromatograms.

(B) Aminoaciduria in Rats after Treatment with gBBE

While the chronic experiments described in Section (*A*) were in progress, it was observed that there was an intense ninhydrin reaction on the marker

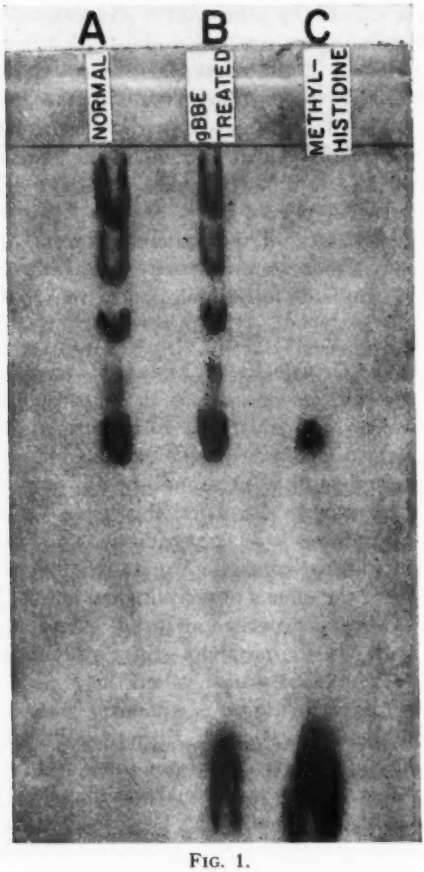


FIG. 1.

strips of the developed chromatograms from the urine of gBBE-treated rats. This reaction was not restricted to any one band on the chromatogram, but was unevenly distributed throughout the paper strip. Previous work by Ames *et al.* (1) and Blahd *et al.* (2) demonstrated that, in patients with muscular dystrophy, there was excessive aminoaciduria. In general these workers found that the excretion of most of the amino acids was increased and methylhistidine was found to be among them.

In our experiments, urine samples of normal (saline-treated) and gBBE-treated rats were collected and processed as described above. The 5-mg and 50-μg dosages of gBBE were also administered to the rats as previously described. Paper chromatographic analysis of the concentrated urine samples was performed in one dimension only with the propanol–water system. Typical results of such an experiment are shown in Fig. 2. It is evident that the rat which received 5-mg doses of gBBE excreted larger amounts of ninhydrin-

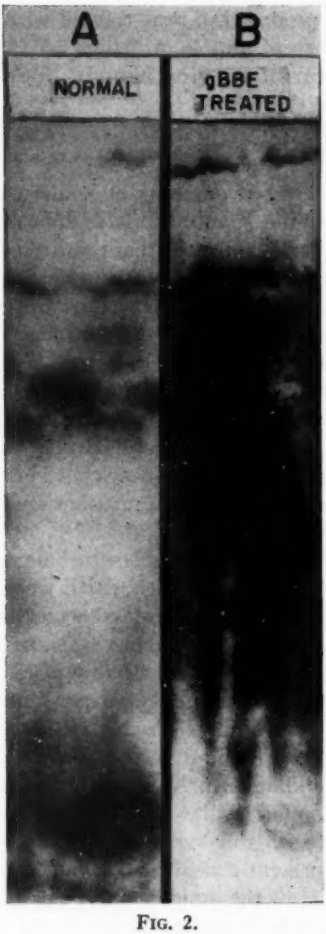


FIG. 2.

positive material than did the saline-treated normal animal. Rats receiving 50 μg gBBE did not show evidence of excessive aminoaciduria when compared with the controls, until the end of the 3rd week of treatment. The aminoaciduria was more intense, however, at the end of the 4th week than that observed at the end of the 3rd.

In these chronic experiments and in other studies (Hosein and Perel, unpublished) we have observed that chronic intramuscular injections of 10 mg gBBE three times daily into rats, for 10 days, caused a gross disappearance of the musculature and its replacement by tissue which, from its appearance, we describe as "fatty tissues". Ether extraction of this tissue has revealed that the "fat content" was increased by more than 50% as compared with that normally present in muscle. From the results presented in this paper, we feel

that the aminoaciduria produced in rats, treated with gBBE, probably results from excessive breakdown of muscle proteins, since macroscopic examination of other tissues did not reveal any gross abnormalities, e.g., "fatty tissue", such as that observed only in the treated muscles.

Discussion

The appearance of increased amounts of amino acids in the urine of rats treated with gBBE and the simultaneous disappearance of muscle with its replacement by "fatty tissue" indicated that this drug promotes in some manner the catabolism of muscle proteins. Experiments in our laboratory (Hosein and Powell, unpublished) have shown that the intramuscular injections of gBBE into rats caused a loss of certain glycolytic enzymes from the injected muscle, while similar enzymes from liver were unaffected. Other studies in this laboratory (14) have also shown that there is pentosuria associated with the effects of gBBE injections and that, in vitro, gBBE significantly inhibits the hexose monophosphate shunt in the erythrocyte (Hosein and Brownstone, unpublished). From these results it would appear that both the glycolytic pathway and the hexose monophosphate shunt in muscle tissue of animals treated with gBBE could be impaired.

As a consequence of these metabolic disturbances, the energy-producing systems in the muscle of rats treated with gBBE would be expected to be low. The processes which could initiate the breakdown of protein are unknown, but the low tissue content of energy-producing systems, such as ATP, phosphocreatine, and enzymes in glycolysis, is probably a significant factor.

On the other hand, it also appears likely that metabolic systems, requiring ATP as a cofactor, could be inhibited by gBBE. In other work (Hosein and Smart, unpublished) we have found that in vitro, the synthesis of anserine from β-alanine and methylhistidine with ATP and anserine synthetase is strongly inhibited by gBBE. Similar results were obtained by Hosein and Boisvert (unpublished), who studied the synthesis of glutamine, another reaction requiring ATP. At the present time, it is not known whether inhibition of the synthesis of the dipeptide or the amide could be extended to the synthesis of polypeptides. Should this be found to be so, it may also help to explain the aminoaciduria observed in the experiments described in this paper. These studies are now in progress.

Acknowledgment

This work was supported by a grant from the Muscular Dystrophy Association of Canada, to whom we express our appreciation.

References

1. S. R. Ames and H. A. Risley. Proc. Soc. Exptl. Biol. Med. **68**, 131 (1948).
2. W. H. Blahd, A. Bloom, and W. Drell. Proc. Soc. Exptl. Biol. Med. **90**, 704 (1955).
3. S. P. Datta and H. Harris. Nature, **168**, 296 (1951).
4. I. McManus. J. Biol. Chem. **225**, 325 (1957).
5. E. A. Hosein and M. Smart. Can. J. Biochem. and Physiol. **38**, 569 (1960).
6. S. E. Severin, N. P. Meshkova, and N. I. Rasumovskaya. Doklady Akad. Nauk S.S.S.R. **103**, 871 (1955).

7. J. G. REINHOLD and G. R. KINGSLEY. J. Clin. Invest. **17**, 377 (1938).
8. N. R. JONES. Biochem. J. **57**, xxiv (1954).
9. I. M. WEINSTOCK, A. D. GOLDRICH, and A. T. MILHORAT. Proc. Soc. Exptl. Biol. Med. **91**, 302 (1956).
10. H. M. HINES. Proc. 1st and 2nd Med. Conf. Muscular Dystrophy Assoc. America. *Edited by* A. T. Milhorat. New York. 1951–1952. p. 89.
11. E. FISCHER and V. W. RAMSEY. Am. J. Physiol. **145**, 571 (1946).
12. J. C. DREYFUS and G. SCHAPIRA. Compt. rend. soc. biol. **147**, 1145 (1953).
13. J. C. DREYFUS, G. SCHAPIRA, and F. SCHAPIRA. J. Clin. Invest. **33**, 794 (1954).
14. E. A. HOSEIN. To be published.
15. E. A. HOSEIN. Protides of the biological fluids. *Edited by* H. Peeters. Elsevier, Amsterdam. 1960. p. 275–280.

THE EFFECTS OF ADRENALECTOMY AND CORTISONE ON ZINC METABOLISM IN THE SEX GLANDS AND ADRENAL OF THE MALE RAT[1]

ALLAN D. RUDZIK AND BERNARD E. RIEDEL

Abstract

The effects of adrenalectomy and of cortisone treatment on the uptake of Zn^{65} and the concentration of zinc were observed in whole blood, adrenals, testis, and dorsolateral and ventral prostates of the rat. Cortisone was administered to both normal and adrenalectomized animals either as a single dose or as a daily dose over a period of 14 days. Zinc concentration was decreased in adrenalectomized animals in the blood and dorsolateral prostate. The incorporation of Zn^{65} was decreased after adrenalectomy in the testis and dorsolateral prostate. Daily treatment with cortisone resulted in recovery to normal of the zinc concentrations and uptake of Zn^{65}.

The adrenal gland was found to contain a relatively large amount of zinc and this concentration was doubled with the chronic administration of cortisone. A single dose of cortisone had no effect on the concentration of zinc but caused an increase in the uptake of Zn^{65} in the adrenal.

Introduction

Studies on the distribution of the trace element zinc by Mawson and Fischer in 1951 (1) showed that the concentration in the prostate gland was greater than in any other organ in the rat, rabbit, and man. Gunn, Gould, Ginori, and Morse (2) reported that the dorsolateral prostate concentrated Zn^{65} from 15 to 25 times more than any other organ. These workers also showed in the rat that Zn^{65} uptake by the posterior prostate increased with age of the animal to a maximum at about 12–15 weeks although the uptake in other tissues was not influenced by age.

Gunn and Gould (3) have also studied the effects of androgens and estrogens on the selective uptake of Zn^{65} by the dorsolateral prostate. They observed that androgens could prevent the decrease of Zn^{65} uptake observed after castration. Estrogens were found to be equally effective but the dose level was very critical. Both sex hormones caused an inhibition of Zn^{65} uptake in normal animals. Gonadotrophins and testosterone propionate administered to immature rats caused a marked increase in the size, zinc concentration, and rate of Zn^{65} uptake by the dorsolateral prostate (4).

It was thought of interest to investigate further the effects on zinc metabolism of alterations in hormone balance related to the adrenal gland.

Methods

Male albino rats of the Wistar strain,* weighing from 240–260 g, corresponding to 5 to 6 months of age, were used. Adrenalectomy was performed by the dorsal route and the animals were maintained on "Miracle" dog food pellets†

[1]Manuscript received in original form December 10, 1959, and, as revised, May 9, 1960.
Contribution from the Faculty of Pharmacy, University of Alberta, Edmonton, Alberta. This work was supported by a grant from the National Research Council.

* E. G. Steinhilber & Co. Ltd.

†Ogilvie Flour Mills Co. Ltd.

and saline 1% ad libitum. Intact control animals from the same group of rats were kept under the same conditions of light, temperature, and diet, with the exception that water was given instead of saline. The mean weight of the adrenalectomized animals was not changed at the end of the 14-day period of adrenalectomy as compared with that for intact animals.

Two series of experiments were carried out. In one series intact and adrenalectomized animals were injected daily with 12.5 mg of cortisone acetate (Cortone, Merck, Sharpe & Dohme), administered intraperitoneally, for a period of 14 days, and killed 24 hours after the last dose. In the second series a single dose of 25 mg of cortisone acetate was injected intraperitoneally and the animals killed 24 hours later. In each of these series both intact control animals and adrenalectomized control animals were used.

Twenty-four hours before killing 70 μc Zn^{65}‡ was injected intravenously via the external jugular vein while the animals were under ether anesthesia. The animals were starved but were allowed water or saline ad libitum during the 24 hours before killing. At the time of killing the rats were anesthetized with ether and killed by exsanguination.

A sample of blood, the adrenals, one testis, and the ventral and dorsolateral prostate glands were removed, weighed, ashed, and analyzed for zinc concentration according to the dithizone method of Vallee and Gibson (5). Readings were made on a Beckman Model B spectrophotometer at wavelengths of 515 and 625 mμ. The zinc–dithizone complex was extracted with dilute sulphuric acid and the Zn^{65} activity determined in a well scintillation counter (Nuclear-Chicago) attached to a decade scaler (Tracerlab).

Using the Beckman Model B spectrophotometer and taking readings at 515 and 625 mμ, it was found that the K value in the formula proposed by Vallee and Gibson (5) was $5.94 \pm .05$. Standard solutions of zinc prepared to contain 2 to 50 micrograms of zinc were found to give a linear relationship when plotted against the corrected optical density measured at 515 mμ.

Counting of the radioactivity was held to an error of 2% or less in each sample.

Definition of Terms

Zinc concentration is reported as micrograms per gram wet weight. Specific activity (S/A) is defined as counts per minute/microgram of zinc.

Corrected specific activity (corr. S/A) is specific activity $\times$ weight of animal $\times 10^{-3}$ and is used to correct the activity of the blood for animal weight differences.

Relative specific activity (rel. S/A) is defined as (specific activity of the tissue/specific activity of the blood) $\times 10^2$.

Relative specific activity is used to correct for any differences due to weight of animals within the experimental groups. Significance is indicated only when the values are within 95% confidence limits.

‡Zn^{65} was obtained as a specially irradiated sample from Dr. C. A. Mawson, Chalk River, Ontario, and had a specific activity of approximately 1 mc per mg.

Results

The results obtained are presented in Table I and Table II.

(a) Whole Blood

Adrenalectomy caused a significant decrease in the concentration of zinc in whole blood (Table I). A dose of 12.5 mg of cortisone administered daily for 14 days to adrenalectomized animals resulted in a return to normal in the zinc concentration. A single dose of 25 mg of cortisone given to intact animals caused a reduction in the concentration of zinc in the blood (similar to that of adrenalectomy). Table II shows that the specific activity of the blood, corrected for differences in animal weights, was not altered by adrenalectomy but the administration of cortisone in both single dosage and daily dosage for 14 days resulted in a marked increase.

The intact animals used all weighed 240 g $\pm$ 5 g. Animals from the same shipment were used for adrenalectomy. In the animals, both intact and adrenalectomized, treated with a single dose of cortisone there was no weight change. In the adrenalectomized animals treated daily for 14 days with cortisone there was an average increase in weight of 5 g from the weight at adrenalectomy although there were two animals which lost weight and five which showed no increase in 14 days. Intact animals treated daily for 14 days with cortisone showed an average increase in weight of 5 g.

The observed increase in the concentration of zinc and the specific activity in blood in chronic cortisone-treated adrenalectomized animals suggests either a retention of zinc in the blood stream, or an increased mobility of zinc in the body. Since an increase in specific activity was also noted, without a concurrent increase in the zinc concentration, in adrenalectomized animals receiving a single dose of cortisone, it would appear that there was an increased utilization by the body of the zinc in this compartment.

(b) Ventral Prostate

The concentration of zinc in the ventral prostate was found to be low and was not altered by any of the treatments used (Table I). Borderline increases in the specific activity, relative to the specific activity of the blood, were noted in both intact and adrenalectomized animals when treated with daily doses of cortisone (Table II). A similar increase was observed in adrenalectomized animals after a single dose of cortisone.

(c) Dorsolateral Prostate

The dorsolateral prostate was found to have a concentration of zinc about 10 times that of the ventral prostate (Table I). Table III shows the actual content of zinc present in the glands of cortisone-treated animals compared with the controls in each case. Adrenalectomy showed a marked decrease in the zinc concentration (Table I), which would appear to be entirely due to an increase in the wet weight of the tissue without a concomitant increase in zinc (Table III). It was observed that the dorsolateral prostate became mushy in animals which had been adrenalectomized. The administration of cortisone to intact animals caused an increase in weight of the gland but with

TABLE I

Effects of adrenalectomy and the administration of cortisone on zinc concentration in μg/g wet weight

Group of animals	No. of animals	Blood	Ventral prostate	Dorsolateral prostate	Testis	Adrenals
Intact	24	10.8±2.0[a]*	19.6±4.2	190.0±41.3*	23.3±3.6	59.2±11.5*
Intact + cortisone (chronic)	10	11.5±3.2	18.6±4.5	211.1±32.0	28.4±4.9	106.7±15.2*
Intact + cortisone (acute)	7	5.5±0.3*	17.1±4.2	212.3±20.3	22.9±1.4	66.8± 8.9
Adrenalectomized	9	6.6±1.8**	17.0±6.2	139.0±23.0**	22.1±3.3*	
Adrenal. + cortisone (chronic)	10	12.0±3.5**	17.8±4.2	195.0±35.0**	28.4±4.9*	
Adrenal. + cortisone (acute)	8	4.6±2.1	14.0±2.0	226.0±41.0**	23.5±2.0	

[a]Standard deviation of the mean.
* or ** indicate significance at the 95% confidence level. Comparison is made in each vertical column between the asterisks.

TABLE II

Effects of adrenalectomy and the administration of cortisone on Zn^{65} incorporation

Group of animals	No. of animals	Blood (corr.S/A)[a]	Ventral prostate (rel. S/A)[b]	Dorsolateral prostate (rel. S/A)	Testis (rel. S/A)	Adrenals (rel. S/A)
Intact	24	272 ± 38.0[c]	112.0 ± 23.0*	249 ± 52.0*	86.0 ± 20.0*	131.0 ± 36.0*
Intact + cortisone (chronic)	10	253 ± 41.0	144.0 ± 23.9*	269 ± 54.0	88.0 ± 23.5	116.0 ± 31.0
Intact + cortisone (acute)	7	294 ± 11.0	121.8 ± 5.0	215.6 ± 22.0	66.0 ± 5.6*	170.0 ± 32.0*
Adrenalectomized	9	260 ± 27.0*	100.0 ± 26.0**	206.0 ± 43.0**	69.0 ± 14.0	
Adrenal. ± cortisone (chronic)	10	373 ± 26.0*	129.0 ± 33.0**	301.0 ± 37.5**	87.0 ± 31.0	
Adrenal. + cortisone (acute)	8	356 ± 19.0*	125.0 ± 13.0**	186.2 ± 11.0	57.4 ± 6.3	

[a]Corrected specific activity = specific activity × weight of animal × 10^{-6}.
[b]Relative specific activity = (specific activity of tissue/specific activity of blood) × 10^3.
[c]Standard deviation of the mean.
* or ** indicate significance at the 95% confidence level. Comparison is made in each vertical column between the asterisks.

TABLE III

Zinc content of the dorsolateral prostate gland as compared with the weight of the gland

Group of animals	No. of animals	Zn, μg	Weight of gland, mg
Intact	24	26.26±5.7	138.2±28.5
Intact + cortisone (chronic)	10	33.46±4.9	171.6±45.5
Adrenalectomized	9	22.41±3.4	161.2±32.5
Adrenal. + cortisone (chronic)	10	30.69±5.5	135.8±50.0

no significant change in the zinc concentration; thus some increase in the actual amount of zinc did appear to occur (Table I and Table III). The administration of cortisone to the adrenalectomized animals caused a return to normal in the weight of the gland (Table III) and an expected change in the zinc content was noted.

The relative specific activity was decreased by adrenalectomy as shown in Table II. Treatment with cortisone daily for 14 days resulted in a return to a high level, but no change was observed after a single dose of cortisone.

(d) Testis

The concentration of zinc in the testis was similar to that in the ventral prostate (Table I). The increase in concentration reported was due to a change in weight of the tissue. The decrease in relative specific activity observed in intact animals (Table II) after a single dose of cortisone was significant but cannot at the moment be explained.

(e) Adrenal Gland

The concentration of zinc in the adrenal gland of intact animals was found to be relatively high compared with blood, 59.2 μg/g wet weight as compared with 10.8 μg/g wet weight (Table I). Treatment with cortisone over a period of 14 days resulted in a dramatic increase in the zinc concentration in the adrenal to 106.7 μg/g wet weight. Adrenal hormones from an external source have a profound effect on the adrenal glands. Histological examination of the glands was not undertaken. The weight of the glands, however, did not alter sufficiently to account for the large increase in zinc concentration. The weight of the glands of intact animals was 0.0365±0.0110 g and the weight of the glands of intact animals treated with cortisone (daily) was 0.0328±0.0063 g.

A single dose of cortisone caused no change in the zinc concentration. However, with a single dose of cortisone the relative specific activity was markedly increased whereas no change was observed in the chronic cortisone-treated animals.

It is surprising that there was not an increase in the Zn^{65} uptake by the adrenal gland in the chronic cortisone-treated animals. It is possible that such an increase was masked by the very large increase observed in the zinc concentration in these animals. This possibility is strengthened by the fact that the relative specific activity observed after chronic treatment with cortisone was the same as that of the intact animals but the concentration of zinc was

much higher so that the actual activity must of necessity have been much higher than that of intact animals.

Discussion

Further evidence is presented to confirm the observations of Mawson and Fischer (1) and Gunn *et al.* (2) that the dorsolateral prostate has a selective ability to concentrate zinc as measured by Zn^{65} uptake. The ventral lobe of the prostate is quite different and it would seem that these two lobes are functionally different organs. Others have noticed differences in fructose and citric acid content (6) and acid and alkaline phosphatase activity (1) in these portions of the gland.

Zinc has conclusively been proved to be present in the carbonic anhydrase molecule (7). This cannot be its only role in the dorsolateral prostate, however, since the zinc to carbonic anhydrase ratio has been found to be 5 to 10 times greater in this tissue than in the red blood cell where all the zinc is known to be associated with this enzyme (8). Zinc has been found to be associated with certain other enzymes such as alcohol, glutamic and lactic dehydrogenases (9, 10, 11), phosphatases (8), and carboxypeptidase (12).

Gunn *et al.* (2) have suggested that the degree of uptake of Zn^{65} by the posterior prostate is an indication of the functional state of the gland. It would thus appear that the functional capacity of the dorsolateral prostate is severely affected by adrenalectomy. A single dose of cortisone given to adrenalectomized animals caused complete recovery of the zinc concentration to the normal level. The relative specific activity was not increased. After daily administration of cortisone for a period of 14 days' recovery to the normal levels was observed in the zinc concentration and a recovery to a supranormal level was observed in the Zn^{65} level. These changes suggest that the dorsolateral prostate is dependent upon normal adrenal activity for proper functional activity.

The marked increase in zinc concentration in the adrenal gland of intact animals treated daily with cortisone was particularly interesting. It would appear that there is a relationship between zinc concentration and the functional activity of this gland. A further evaluation of the significance of zinc in the adrenal gland is being undertaken with a study of hypophysectomized animals.

References

1. C. A. Mawson and M. I. Fischer. Nature, **167**, 859 (1951).
2. S. A. Gunn, T. C. Gould, S. S. Ginori, and J. G. Morse. Proc. Soc. Exptl. Biol. Med. **88**, 556 (1955).
3. S. A. Gunn and T. C. Gould. Endocrinology, **58**, 443 (1956).
4. M. J. Millar, P. V. Elcoate, and C. A. Mawson. Can. J. Biochem. and Physiol. **35**, 865 (1957).
5. B. L. Vallee and J. G. Gibson. J. Biol. Chem. **176**, 435 (1948).
6. G. F. Humphrey and T. Mann. Nature, **161**, 352 (1948).
7. D. Keilin and T. Mann. J. Biochem. **34**, 1163 (1940).
8. C. A. Mawson and M. I. Fischer. Arch. Biochem. Biophys. **36**, 485 (1952).
9. B. L. Vallee and F. L. Hoch. J. Am. Chem. Soc. **77**, 821 (1955).
10. B. L. Vallee and W. E. C. Wacker. J. Am. Chem. Soc. **78**, 1771 (1956).
11. B. L. Vallee, S. J. Adelstein, and J. G. Olsen. J. Am. Chem. Soc. **77**, 5196 (1955).
12. B. L. Vallee and A. Neurath. J. Biol. Chem. **217**, 253 (1955).

THE CHROMATOGRAPHIC SEPARATION OF HOMOLOGOUS PHOSPHATIDES[1]

ERICH BAER, DMYTRO BUCHNEA, AND TIBOR GRÓF

Abstract

The feasibility of separating homologous phosphatides by paper chromatography has been established. Evidence is presented for the chromatographic separation of members of homologous series of glycerolphosphatides differing by eight, four or two carbon atoms per fatty acid substituent.

The separation of fats and phosphatides into their individual components has challenged chemists and biochemists for more than a century. It offers some of the most difficult problems in the separation of natural products. The earlier procedures for the separation of either fats or phosphatides made use of the subtle differences in the solubility of their components in organic solvents. In recent years these methods have been supplemented by countercurrent and chromatographic procedures. By a judicial combination of these procedures a fairly satisfactory separation of naturally occurring mixtures of phosphatides into their various classes is now possible. Furthermore there are indications that a chromatographic separation of these into species is also within the realm of possibility. Rhodes and Lea (1), and Hanahan, Dittmer, and Warashina (2), investigating the separation of egg and liver phosphatides, respectively, by column chromatography on silicic acid observed that the faster-running lecithin fractions had a higher degree of unsaturation than the slower-moving material. While the isolation of pure individual phosphatides from biological sources has been achieved in a few instances, these cases are rare and were made possible only by favorable circumstances.

A tremendous amount of work has been expended on studies concerning the biological functions of the phosphatides, using products now known to have been non-homogeneous. The results have not been too satisfactory. Obviously, these studies would be greatly facilitated by an exact knowledge of the individual phosphatides involved in the biological reactions, as only then can the qualitative and quantitative changes associated with these functions be satisfactorily studied. This requires methods for their isolation which not only permit the separation of natural phosphatides into classes and species, but also the separation of these into their individual components. An investigation into the possibility of separating homologous phosphatides by chromatographic procedures therefore seemed highly desirable. To obtain definitive results, however, known mixtures of pure phosphatides would have to be used. Suitable substrates for this purpose have become available by the recent synthesis of the dihexanoyl-, dioctanoyl- and didecanoyl-L-α-lecithins (3) and of dihexanoyl-L-α-cephalin (4). These phosphatides, together with their higher homologues, also synthesized in this laboratory (5–9), form two homologous series whose

[1]Manuscript received March 8, 1960.
Contribution from the Subdepartment of Synthetic Chemistry in Relation to Medical Research, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.

members differ widely in the length of the carbon chains (C_6–C_{18}) of their fatty acid substituents. No comparable series of phosphatides of biological origin are known at the present time. For the initial experiments we selected two homologous pairs of phosphatides whose fatty acid substituents differed by eight carbon atoms, namely dihexanoyl- and dimyristoyl-L-α-cephalin (DHC, DMC), and dihexanoyl- and dimyristoyl-L-α-lecithin (DHL, DML). Each of these pairs should be readily separable if chromatographic procedures are to be of any value in the separation of homologous phosphatides. The chromatographic separation of the homologous pairs was carried out according to the procedure of Marinetti and Stotz (10), viz. on silicic-acid-impregnated paper using a solvent system consisting of diisobutyl ketone, acetic acid, and water. To locate the positions of the cephalins, the paper strip was sprayed with a solution of ninhydrin in acetone and lutidine. The lecithins were located by spraying the paper with a mixture of perchloric acid, hydrochloric acid, and molybdic acid, and reducing the phosphomolybdic acid complex with hydrogen sulphide according to the method of Hanes and Isherwood (11). When an attempt was made to locate the DHL and DML by a 1-minute immersion of the paper strip in an aqueous solution of rhodamine 6 G, and visual inspection of the chromatogram in ultraviolet light, only the DML could be detected. The DHL, being highly water-soluble, had been removed in the rhodamine bath. This method for locating phosphatides obviously is to be avoided if there is a suspicion that water-soluble phosphatides are present.

Figures 1 and 2 show that both pairs of homologues have been cleanly separated. This is, to our knowledge, the first demonstration of a separation of homologous phosphatides by paper chromatography or by any other means.

In these and later experiments the phosphatides used by us were chromatographically homogeneous compounds, except in one experiment, where a fairly old preparation of L-α-(dimyristoyl)cephalin containing traces of lysocompounds was used. On parallel runs of this particular preparation of DMC with DHC it was observed that the lysomyristoylcephalin(s) appeared on the chromatogram in the same region as DHC. This is of interest as it suggests that components of mixtures of naturally occurring phosphatides that have previously been identified as lysocompounds on the basis of their chromatographic behavior may well have been phosphatides with fatty acid substituents of shorter chain length. It also serves as a warning that chromatographic procedures should be used only in conjunction with other methods permitting the unequivocal identification of phosphatides.

The fairly wide gaps separating the hexanoyl and myristoyl compounds (Figs. 1 and 2) suggested that it should be possible to separate by this method homologues of both series of compounds that differ by less than eight carbon atoms per fatty acid substituent. To test this possibility we investigated the separation of a mixture consisting of dihexanoyl-, didecanoyl- and dimyristoyl-L-α-lecithin. The results of this experiment are shown by Fig. 3. Again the lecithins have been distinctly separated. However, the C_{10}- and C_{14}-lecithins are spaced fairly close together, and it appears that the separation of lecithins differing by four carbon atoms per fatty acid constitutes the limit of resolution

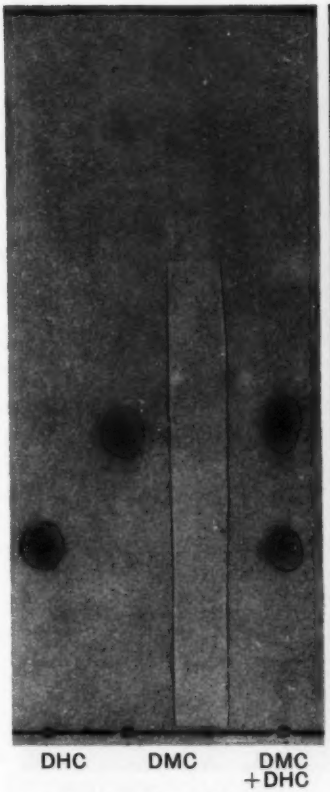


FIG. 1. Separation of DHC (30 γ) and DMC (40 γ).

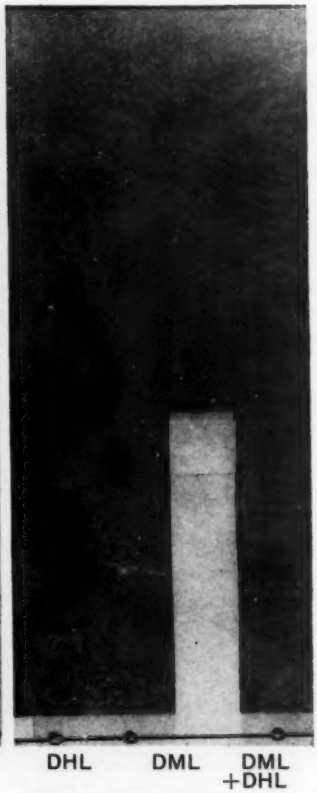


FIG. 2. Separation of DHL (30 γ) and DML (40 γ).

that can be achieved for saturated α-lecithins with fatty acid substituents of more than 10 carbon atoms by one-dimensional chromatography and employing the solvent system: diisobutyl ketone, acetic acid, and water. It is conceivable, however, that a separation of the higher members of the homologous series of α-lecithins differing by fewer than four carbon atoms may be accomplished by using other solvent systems or two-dimensional chromatography. These possibilities have not yet been investigated. On the other hand the fairly wide gap separating the C_6- and C_{10}-lecithins (Fig. 3) indicated that members of this series differing by fewer than four carbon atoms should be separable if their fatty acid substituents have not more than 10 carbon atoms. This possibility was tested with a mixture of phosphatides consisting of dihexanoyl-, dioctanoyl- and didecanoyl-L-α-lecithin. A complete separation of the three lecithins was achieved (Fig. 4). Whether or not the corresponding trio of homologous α-cephalins are also separable by this procedure could not be tested as the necessary materials are not yet available. A comparison of Figs. 1 and 2 leaves little doubt, however, that this too should be possible.

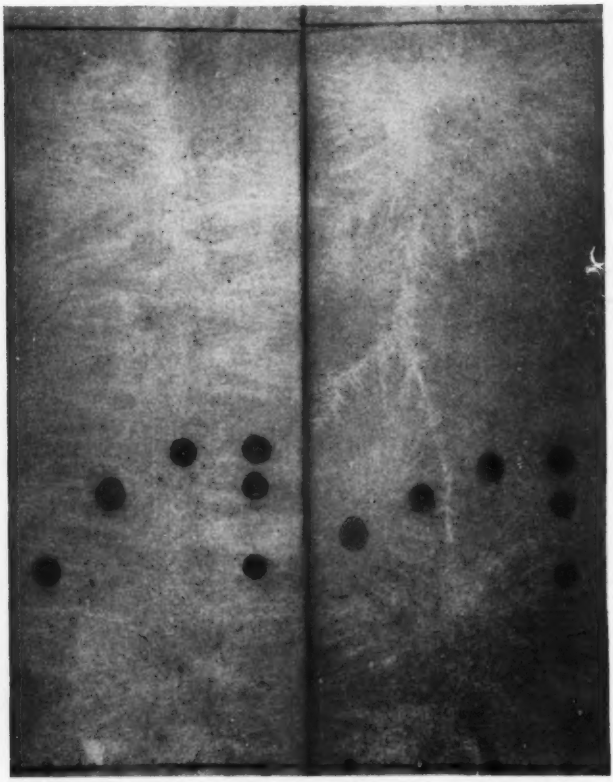


FIG. 3. Separation of DHL (30 γ), DDL (37.5 γ), and DML (40 γ).

FIG. 4. Separation of DHL (30 γ), DOL (35 γ), and DDL (37.5 γ).

Now that the separation of homologous phosphatides by paper chromatography has been shown to be feasible, the means exist for more successful studies of the composite nature of the highly complex mixtures of biological phosphatides.

Experimental

Separation of Two Cephalins Differing by Eight Carbon Atoms per Fatty Acid. L-α-(Dihexanoyl)cephalin and L-α-(Dimyristoyl)cephalin

A strip (11×14 cm) of silicic-acid-impregnated Whatman No. 1 filter paper was spotted as indicated in Fig. 1 with 10 μl of a solution containing 3.0 mg of DHC in 1.0 ml of chloroform (solution I), 10 μl of a solution containing 4.0 mg of DMC in 1.0 ml of chloroform (solution II), and with 10 μl each of solutions I and II. The chromatogram was developed by the ascending

technique in a cylindrical glass chamber (13×46 cm) using a solvent mixture composed of diisobutyl ketone/glacial acetic acid/distilled water, 40/25/5 by volume (10). After 20 hours the paper strip was air-dried and the cephalins were located by spraying with a 0.3% solution of ninhydrin in acetone/lutidine, 95/5 by volume.

Separation of Two Lecithins Differing by Eight Carbon Atoms per Fatty Acid. L-α-(Dihexanoyl)lecithin and L-α-(Dimyristoyl)lecithin

The chromatographic separation of the two lecithins was carried out as described above for the cephalins, using 10 μl each of their chloroform solutions containing 3.0 mg of DHL and 4.0 mg of DML per 1.0 ml of solvent. After 20 hours the paper strip was dried in an oven at 70°, and the lecithins were located according to the method of Hanes and Isherwood (11). The results are shown by Fig. 2.

Separation of Mixtures of Three Lecithins Each, Whose Vicinal Homologues Differ in One Series by Four and in the Other by Two Carbon Atoms per Fatty Acid

The separation of these two mixtures, one consisting of dihexanoyl-, didecanoyl- and dimyristoyl-L-α-lecithin, the other of dihexanoyl-, dioctanoyl- and didecanoyl-L-α-lecithin, and the determination of the positions of the lecithins on the chromatograms were carried out as described above for the mixture of DHL and DML. The chloroform solutions of dioctanoyl- and didecanoyl-L-α-lecithin contained 3.50 mg and 3.75 mg of the respective lecithins per 1.0 ml of solvent. The results are shown by Figs. 3 and 4.

Acknowledgment

The authors express their gratitude to the Multiple Sclerosis Society of Canada for generous grants in support of this and related work.

References

1. D. N. Rhodes and C. H. Lea. *In* Biochemical problems of lipids. *Edited by* Popjak and LeBreton. Interscience Publishers, Inc., New York. 1956. p. 73.
2. D. J. Hanahan, J. C. Dittmer, and E. Warashina. J. Biol. Chem. **228**, 685 (1957).
3. E. Baer and V. Mahadevan. J. Am. Chem. Soc. **81**, 2494 (1959).
4. E. Baer and T. Gróf. Can. J. Biochem. and Physiol. This issue.
5. E. Baer, J. Maurukas, and M. Russell. J. Am. Chem. Soc. **74**, 152 (1952).
6. E. Baer and M. Kates. J. Am. Chem. Soc. **72**, 942 (1950).
7. E. Baer and J. Maurukas. J. Am. Chem. Soc. **74**, 158 (1952).
8. E. Baer. J. Am. Chem. Soc. **75**, 621 (1953).
9. E. Baer. Can. J. Biochem. and Physiol. **35**, 239 (1957).
10. G. V. Marinetti and E. Stotz. Biochim. et Biophys. Acta, **21**, 168 (1956).
11. C. S. Hanes and F. A. Isherwood. Nature, **164**, 1107 (1949).

SYNTHESIS OF L-α-CEPHALINS CONTAINING FATTY ACIDS OF SHORTER CHAIN LENGTH

WATER-SOLUBLE GLYCEROLPHOSPHATIDES II[1]

ERICH BAER AND TIBOR GRÓF

Abstract

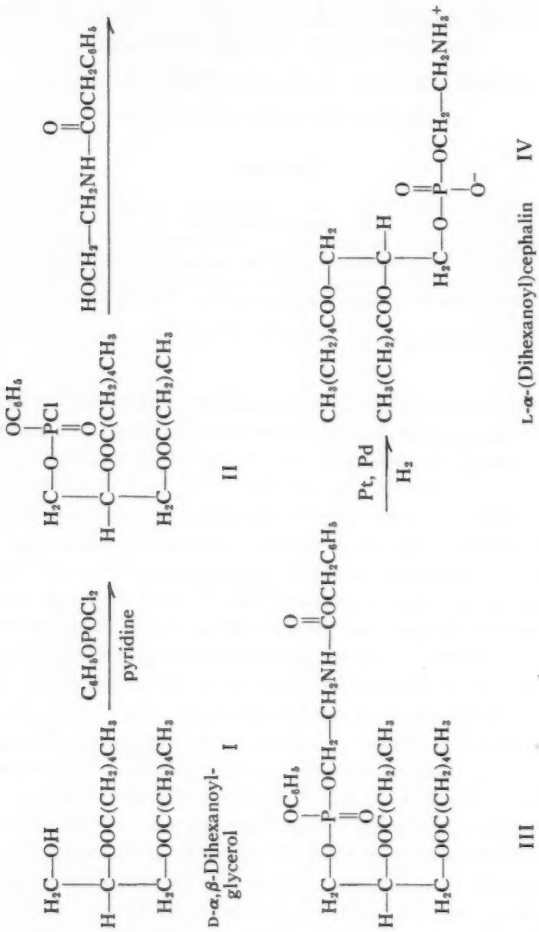
L-α-(Dihexanoyl)cephalin has been synthesized by the phosphorylation of D-α,β-dihexanoylglycerol with phenylphosphoryl dichloride and pyridine, esterification of the reaction product, viz. dihexanoyl-L-α-glycerylphenylphosphoryl chloride, with N-carbobenzoxyethanolamine, and simultaneous removal of the protective groups of dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine by catalytic hydrogenolysis. The L-α-(dihexanoyl)cephalin is soluble in water.

Infrared evidence supports the inner-salt structure of cephalins in chloroform solution.

A year ago, Baer and Mahadevan (1) reported the synthesis of L-α-lecithins with fatty acid substituents of shorter chain length than any fatty acid hitherto found to be present in naturally occurring glycerolphosphatides. These lecithins containing capric (C_{10}), caprylic (C_8), and caproic (C_6) acid substituents, in contrast to their higher homologues, were found to be highly soluble at room temperature in methanol, ethanol, and acetone, and the last one also in water. This was of interest as it suggested that lecithins with fatty acid substituents of 10 carbon atoms or less, if they do occur in nature, may have escaped recognition because they would be lost in conventional fractionation procedures. Whether or not this applies also to other types of phosphatides with similar fatty acid substituents has yet to be established. For this and other reasons the synthesis of glycerolphosphatides with fatty acid substituents of shorter chain length has been extended to the cephalins. The present paper deals with the preparation of L-α-(dihexanoyl)cephalin. It was obtained by the procedure of Baer, Maurukas, and Russell (2) for the synthesis of saturated α-cephalins (Reaction Scheme), except that the separation of the phosphorylation products, which possess very similar solubility properties, was accomplished by means of as ilicic acid column. D-α,β-Dihexanoylglycerol (I), prepared as described by Baer and Mahadevan (1), was phosphorylated with phenylphosphoryl dichloride and pyridine, and the resulting phenyl ester of the phosphatidic acid chloride (II) was esterified with N-carbobenzoxyethanolamine. The mixture of reaction products was separated by column chromatography on silicic acid, and the dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine (III) was freed of its protective groups by catalytic hydrogenolysis using a mixture of platinum and palladium catalysts. The L-α-(dihexanoyl)cephalin, like the corresponding lecithin, is highly soluble at room temperature in chloroform, methanol, or ethanol, and insoluble in ether or petroleum ether. It differs from the lecithin, however, in that it is only sparingly

[1]Manuscript received March 8, 1960.

Contribution from the Subdepartment of Synthetic Chemistry in Relation to Medical Research, Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.



soluble in acetone or water (approximately 0.4 g per 100 ml of water). Thus it appears that the ethanolamine structure confers much less solubility in water to the phosphatide than does the choline structure. To obtain a cephalin as soluble in water as the dihexanoyllecithin, it would have to have fatty acid substituents with an even shorter chain of carbon atoms. The solubility of the α-dihexanoylcephalin in water is, however, high enough to permit its use as substrate in investigations that are carried out more advantageously in homogeneous aqueous systems.

The molecular rotations of the L-α-(dihexanoyl)cephalin (+42.4°) and its intermediate, dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine (+19.9°), resemble closely those of the corresponding higher homologues (2), and thus confirm the optical purity of both compounds. From the

data available for dihexanoyl-, dimyristoyl-, dipalmitoyl-, and distearoyl-L-α-lecithin a mean molecular rotation for the homologous series of saturated L-α-cephalins of +43.4° is obtained. This value should provide a useful check on the purity of new members of this series.

Figure 1 records the infrared spectra of dihexanoyl-L-α-glycerylphenylphosphoryl N-carbobenzoxyethanolamine (A) and L-α-(dihexanoyl)cephalin (B) in chloroform solution. Both have absorption bands at or near 3.41 μ, 3.50 μ, 5.80 μ, 6.85 μ, 7.10 μ, 7.25 μ, and 9.60 μ. These are also found in the original diglyceride. In addition, the cephalin (B) absorbs at 6.15 μ (primary amine) and the intermediate compound (A) at 6.28 μ, 6.62 μ, and 6.72 μ (C=C skeletal-in-plane vibrations), at 10.45 μ (P—O-phenyl ?), and at 14.55 μ (out-of-plane deformation vibrations, five adjacent free hydrogen atoms). The absence from the spectrum of the cephalin of an absorption band in the usual N—H stretching region (2.85 μ–3.02 μ) favors the concept of an inner-salt

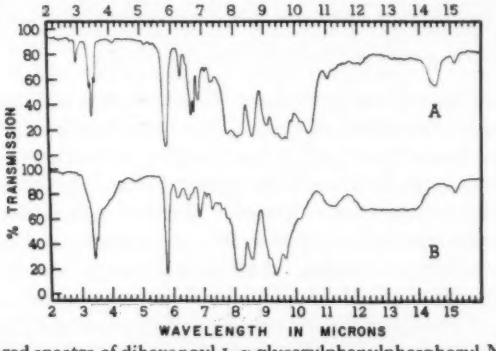


FIG. 1. Infrared spectra of dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine (A) and L-α-(dihexanoyl)cephalin (B). Beckman IR-5 infrared spectrophotometer. Solvent: ethanol-free chloroform. Path of cell: 0.093 mm. Concentrations: (A) 10%, (B) 5%. Band positions and probable assignments: (A) 2.92 μ (N—H stretching), 3.32 μ (C—H stretching), 3.41 μ and 3.50 μ (CH_2-stretching), 5.80 μ (CO-vibration), 6.28 μ, 6.62 μ, and 6.72 μ (C=C skeletal-in-plane vibrations), 6.85 μ (CH_2-deformation frequencies), 7.10 μ (C—H vibrations), 7.25 μ (C—CH_3 deformation frequencies), 9.60 μ (covalent phosphate), 10.45 μ (phenyl), 14.55 μ (five adjacent ring hydrogen atoms). (B) 3.44 μ and 3.54 μ (CH_2-stretching), 5.80 μ (CO-vibration), 6.15 μ (prim. amine deformation frequencies), 6.85 μ (CH_2-deformation frequencies), 7.09 μ (C—H vibrations), 7.3 μ (C—CH_3 sym. deformation frequencies), 9.65 μ (covalent phosphate).
The bands at 3.44, 3.54, 5.80, 6.85, 7.10, and 7.30 μ are present also in α,β-dihexanoylglycerol.

structure (NH_3^+) for the compound in chloroform solution. Amino acids which form inner salts also fail to show absorption in this region (3). The inner-salt structure of the cephalin in chloroform solution receives further support from the fact that the infrared spectrum of the intermediate compound, which is unable to form an inner salt, shows a strong absorption band at 2.92 μ (N—H stretching).

Experimental

Materials

D-α,β-Dihexanoylglycerol was prepared as described by Baer and Mahadevan (1). The N-carbobenzoxyethanolamine was obtained by the procedure of

Chargaff (4) in the modification of Rose (5). Dry and ethanol-free chloroform was prepared immediately before use by distilling chloroform (U.S.P.) over phosphorus pentoxide. Anhydrous pyridine was prepared from the best commercial grade of pyridine available, by refluxing it over barium oxide and distilling it with the exclusion of moisture. The phenylphosphoryl dichloride (b.p. 103°–106° at 9 mm) was prepared by the procedure of Zenftman and McGillivray (6). The palladium catalyst was prepared by the reduction of palladium chloride with formic acid as described by Tausz and Putnoky (7), except that the palladium, after having been washed thoroughly with water, was suspended several times in fresh portions of glacial acetic acid to remove water, and then was stored under glacial acetic acid until used. By avoiding the drying of the reduced palladium, a much more active catalyst was obtained. The platinum catalyst was prepared as described by Adams, Voorhees, and Shriner (8), except that the sodium nitrate was replaced by an equimolecular amount of potassium nitrate (9). The silicic acid (Merck) was sifted using a sieve with 150 meshes per linear inch to remove particles smaller than 100 microns. The hydrogen was produced electrolytically.

*Dihexanoyl-*L*-α-Glycerylphenylphosphoryl-*N*-carbobenzoxyethanolamine*

Into a thoroughly dried 300-ml three-necked flask equipped with an oil-sealed stirrer, dropping funnel, and a T-tube carrying a thermometer and calcium chloride tube were placed 21.1 g (0.10 mole) of freshly prepared and carefully fractionated phenylphosphoryl dichloride. The flask was immersed in a mixture of crushed ice and sodium chloride at −10°, and a solution of 28.8 g (0.10 mole) of D-α,β-dihexanoylglycerol ($[\alpha]_D$ +3.66° in substance) and 9.7 ml (0.12 mole) of anhydrous pyridine in 25 ml of chloroform was added dropwise to the vigorously stirred phosphorylating agent in the course of 1½ hours, care being taken that the temperature of the reaction mixture stayed within the limits of −4° to 0°. After the solution had been added, the cold bath was removed, the stirring was continued, and 1½ hours later 32 ml (0.4 mole) of anhydrous pyridine was added in the course of 30 minutes, followed by the addition of a solution of 19.5 g (0.1 mole) of N-carbobenzoxyethanolamine in 40 ml of anhydrous chloroform over a period of 45 minutes. While the ethanolamine derivative was being added, the reaction mixture was cooled occasionally to keep its temperature below 40°.

Isolation of the Phosphorylation Product

The reaction mixture, after being kept at room temperature for 18 hours, was distilled under reduced pressure (Flash evaporator) at a temperature not exceeding 40° to remove the chloroform and as much of the pyridine as possible. The remaining solid material was taken up in anhydrous ether, the solution was filtered from pyridine hydrochloride, and the filtrate (about 1 liter) was washed in rapid succession with two 150-ml portions each of ice-cold 2 *N* sulphuric acid, water, a saturated solution of sodium bicarbonate, followed by water.[2] The ether layer was dried with anhydrous sodium sulphate, and the solvent was distilled off under reduced pressure from a bath at 35–40°. The remaining

[2]Emulsions were broken by the addition of sodium chloride.

viscous oil was suspended with vigorous shaking in 150 ml of low boiling petroleum ether (b.p. 35°–60°), and the mixture was separated by centrifugation. This procedure was repeated once more with 150 ml of fresh petroleum ether. The remaining oil was kept in a vacuum of 0.02 mm Hg until its weight was constant. The crude dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine, a slightly yellowish oil, weighed 37.74 g (60.7% of theory). It was purified by chromatography on a silicic acid column. The crude material was dissolved in 500 ml of benzene (U.S.P.), and the solution was divided into two equal parts, each of which was passed through a column 45 cm in length and 4.3 cm in width containing 500 g of silicic acid (Merck). Each column was washed with 800 ml of benzene (U.S.P.) followed by 6 liters of a mixture of benzene and anhydrous ether (9:1, v/v). The benzene–ether eluate, containing the desired compound, on distillation under reduced pressure from a bath at 35°–40° gave 23.1 g (61.2% of recovery) of a material that usually was pure enough to be converted directly into the cephalin. If its nitrogen and phosphorus values indicated, however, that a further purification was required, it was reprecipitated from its solution in anhydrous ether (1 ml of solvent per 1.0 g of substance, not more!) by the addition of low-boiling petroleum ether (15 ml per 1.0 g of substance). The mixture was separated by centrifugation and the lower layer was dried to constant weight in a vacuum of 0.02 mm Hg at 35°–40°. The dihexanoyl-L-α-glycerylphenylphosphoryl N-carbobenzoxyethanolamine is readily soluble at room temperature (20°) in methanol, ethanol, acetone, ethyl acetate, ether, chloroform, tetrachloromethane, or benzene, but insoluble in petroleum ether or water. n_D^{20} 1.4984. $[\alpha]_D^{25}$ +3.2° in anhydrous and ethanol-free chloroform (*c*, 10). M_D^{25} +19.9°. Reported (2) for the corresponding myristoyl, palmitoyl, and stearoyl homologues: M_D +21.2°, +21.0°, and +20.1°, respectively.

Anal. Calc. for $C_{31}H_{44}O_{10}NP$ (621.65): C 59.89; H 7.13; N 2.25; P 4.98. Found: C 60.36; H 7.32; N 2.18, 2.17; P 4.98, 4.92.

Dihexanoyl-L-α-Glycerylphosphorylethanolamine

A solution of 2.49 g (4.0 mmoles) of dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine in 65 ml of glacial acetic acid together with 0.4 g of platinum oxide and 0.3 g of palladium black were placed in an all-glass hydrogenation vessel of 250-ml capacity, and the mixture was shaken vigorously in an atmosphere of pure hydrogen at an initial pressure of approximately 50 cm of water until the uptake of hydrogen ceased (approximately 1 hour). After the hydrogen was replaced with nitrogen, the catalyst was removed by centrifugation and washed with small amounts of glacial acetic acid. The solutions were combined, and the acetic acid was distilled off under reduced pressure from a bath at 35°–40°. The residue, weighing 1.56 g (95% of theory), was dissolved in 70 ml of 85% ethanol, the solution was passed rapidly through a column of Amberlite IR-120 (H form) 37 cm long and 2.5 cm wide, and the column was washed with 140 ml of 85% ethanol. The combined effluents were evaporated to dryness under reduced pressure from a bath at 35°–40°, and the residue was kept at this temperature in a vacuum of 0.02 mm until it was dry and free of acetic acid. For further purification the cephalin

(1.45 g) was precipitated from chloroform (3 ml) by the gradual addition of anhydrous ether (90 ml). The mixture, after being left to stand overnight in the ice box, was separated by centrifugation, and the precipitate was washed with anhydrous ether. The remaining material on drying *in vacuo* (0.02 mm) at 35°–40° gave 1.17 g (81% recovery) of L-α-(dihexanoyl)cephalin. $[\alpha]_D^{24}$ +10.4° in anhydrous and ethanol-free chloroform (*c*, 5); M_D +42.8° in chloroform. Reported (2) for the corresponding myristoyl, palmitoyl, and stearoyl homologues: M_D +42.5°, +43.5°, and +44.5°, respectively. The dihexanoylcephalin, a pure white solid, is highly soluble at room temperature (22°) in chloroform, methanol, and ethanol, sparingly soluble in acetone, distilled water (0.4 g/100 ml), water containing 2% acetic acid (0.34 g/100 ml), 5% acetic acid (0.5 g/100 ml), or 5% ethanol (0.42 g/100 ml), and insoluble in ether or petroleum ether.

Anal. Calc. for $C_{17}H_{34}O_8NP$ (411.43): C 49.62; H 8.33; N 3.40; P 7.53. Found: C 49.84; H 8.53; N 3.50; P 7.55.

Both dihexanoyl-L-α-glycerylphenylphosphoryl-N-carbobenzoxyethanolamine and dihexanoyl-L-α-glycerylphosphorylethanolamine were tested by the method of Marinetti and Stotz (10), and were found to be chromatographically homogeneous substances.

Acknowledgment

The authors wish to acknowledge their indebtedness to the Multiple Sclerosis Society of Canada for generous grants in support of this and related work. They wish to thank also Dr. Dmytro Buchnea for his assistance in completing the experimental work, and Professor Maria T. Wishart and her Department of Art as Applied to Medicine for the infrared tracings.

References

1. E. Baer and V. Mahadevan. J. Am. Chem. Soc. **81**, 2494 (1959).
2. E. Baer, J. Maurukas, and M. Russell. J. Am. Chem. Soc. **74**, 152 (1952).
3. L. J. Bellamy. The infra-red spectra of complex molecules. 2nd ed. Methuen and Co. Ltd., London. 1958. p. 237.
4. E. Chargaff. J. Biol. Chem. **118**, 417 (1937).
5. W. G. Rose. J. Am. Chem. Soc. **69**, 1384 (1947).
6. H. Zenftman and R. McGillivray. Chem. Abstr. **45**, 9081 (1951); Brit. Patent No. 651,656.
7. J. Tausz and N. von Putnoky. Ber. **52**, 1573 (1919).
8. R. Adams, V. Voorhees, and R. L. Shriner (*Editors*). *In* Organic syntheses. Collective Vol. I. 2nd ed. John Wiley & Sons, Inc., New York. 1948. p. 463.
9. A. H. Cook and R. P. Linstead. J. Chem. Soc. 952 (1934).
10. G. V. Marinetti and E. Stotz. Biochim. et Biophys. Acta, **21**, 168 (1956).

EVALUATION OF PROTEIN IN FOODS

VI. FURTHER FACTORS INFLUENCING THE PROTEIN EFFICIENCY RATIO OF FOODS[1]

E. J. MIDDLETON, A. B. MORRISON, AND J. A. CAMPBELL

Abstract

Diets containing two levels of protein (10 and 15%) and two levels of fat (10 and 20%) were tested in all possible combinations in a 4-week rat growth assay. Protein was supplied by casein or a mixture of plant proteins. The protein efficiency ratio (P.E.R.) for casein found with a diet containing 10% protein and 10% fat was slightly greater than that found with a diet containing 15% protein and 20% fat, approximating the levels found in the "average" Canadian diet. The value for plant protein was similar under both conditions. Efficiency of carcass protein synthesis was much greater in animals fed casein than in those fed plant protein. With both sources of protein, the efficiency of carcass protein synthesis was similar with diets containing 10% protein and 10% fat to those with 15% protein and 20% fat.

In additional studies, no effect of chlortetracycline or source of carbohydrate on P.E.R. of casein was observed.

It was concluded that P.E.R. determinations carried out on a 10% protein diet furnish a valid estimate of the nutritive value of protein and have several practical advantages.

Although determination of the protein efficiency ratio (P.E.R.) is widely used in the evaluation of protein in foods (1, 2), relatively few studies have been conducted on factors which influence P.E.R. In previous reports from this laboratory (3, 4), it was shown that P.E.R. values are influenced by the strain and sex of the rats used, the age of the rats at the beginning of the experiment, the length of the experimental period, and the level of dietary protein. It was concluded that these factors must be standardized if comparable results are to be obtained in different laboratories. Recently, Harris and Burress (5) criticized the use of diets containing 10% protein in P.E.R. studies. They suggested that the protein level used should be 15%, that found in the average American or Canadian diet, in order to test foods under conditions similar to those under which they are actually eaten. In their studies, Harris and Burress used dietary fat levels of only 5%, whereas the average American or Canadian diet contains approximately 20% fat (6, 7). In the present studies, P.E.R. values found with diets containing 15% protein and 20% fat, the levels in the average diet, were compared with those obtained using the procedure of Chapman *et al.* (3), in which the diets contained 10% protein and 10% fat. The effects of the type of dietary carbohydrate source, and the presence of an antibiotic in the diet, were also briefly investigated.

Experimental

In the studies reported herein, male weanling rats were individually housed in screen-bottom cages kept in an air-conditioned room maintained at 74° to 76° F. Food and water were supplied ad libitum, records kept of the amount of

[1]Manuscript received April 13, 1960.
Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

food consumed by each rat, and the animals weighed individually at weekly intervals for 4 weeks.

In experiment 1, nine comparable groups of 14 Wistar rats each were selected. One group was sacrificed to provide baseline values for studies of nitrogen storage. The remaining eight groups were given diets containing two levels of protein, 10% and 15%, and two levels of corn oil, 10% and 20%, tested in all possible combinations. Two protein sources, casein and a mixture of plant proteins consisting, in per cent, of whole wheat flour 75, wheat gluten 20, and toasted soybean protein 5, were studied. The basal protein-free diet used was that of Chapman *et al.* (3). Protein and fat were added to this diet, at the expense of cornstarch, to give the desired dietary levels. The baseline group of animals was sacrificed by ether anesthesia, exsanguinated, and the eviscerated carcasses were frozen, ground in the frozen state, and analyzed for nitrogen, moisture, and total lipid. In addition, the livers were analyzed for total lipid. Nitrogen was determined by the micro-Kjeldahl procedure, total lipid by the method of Folch *et al.* (8), and moisture by drying an aliquot of each sample at 115° C for 24 hours. At the end of the experiment (28 days), the remaining animals were killed and treated in the same manner.

In experiment 2, two groups of eight rats of the Food and Drug strain received the protein-free diet of Chapman *et al.* (3), supplemented with 10% casein protein (N×6.25), and with either sucrose or cornstarch as the dietary carbohydrate. Two other groups received similar diets containing 100 p.p.m. chlortetracycline (Aureomycin).

The data of the experiments were analyzed by appropriate statistical techniques as outlined by Snedecor (9).

Results and Discussion

Effect of Protein and Fat Levels

P.E.R. values found in experiment 1 are given in Table I. The P.E.R. value for casein was significantly reduced, and that for plant protein was significantly increased, when the dietary protein level was increased from 10 to 15% in the

TABLE I

Effect of dietary protein and fat levels on P.E.R. of casein and a plant protein mixture

Diet	Dietary		Weight gain, g	Food consumption, g	P.E.R.
	Protein, %	Fat, %			
Casein	10	10	98 ± 11*	300	3.25 ± 0.24*
	15	10	131 ± 17	316	2.75 ± 0.19
	10	20	77 ± 13	244	3.17 ± 0.22
	15	20	133 ± 19	290	3.06 ± 0.17
Plant protein	10	10	26 ± 9	200	1.28 ± 0.16
	15	10	56 ± 14	256	1.44 ± 0.13
	10	20	21 ± 5	167	1.24 ± 0.18
	15	20	42 ± 8	202	1.39 ± 0.12

*Standard deviation.

animals given diets containing 10% fat. In the animals receiving 20% fat, no effect of protein level on the P.E.R. of casein was observed, whereas the value for plant protein was significantly greater with animals given 15% protein than with those receiving 10% protein. It is well known (4, 10, 11) that the effect of increasing protein levels on P.E.R. values is dependent on protein quality. Good quality proteins show reduced P.E.R. values as the protein content of the diet is increased from 10 to 15%, whereas the reverse is true for proteins of poorer quality. At the 10% protein level, increasing the level of fat from 10 to 20% had no effect on P.E.R., in animals fed either source of protein. At the 15% protein level, however, increasing the fat level significantly increased the P.E.R. found with casein, but not with the plant protein mixture. The studies show the interrelated effects of protein quality, protein level, and caloric level of the diet on P.E.R. Increasing the caloric level of the diet increased P.E.R. only if the dietary protein level was sufficiently high and of good quality.

It is of interest to compare the P.E.R. values found with diets containing 10% protein and 10% fat, the levels used by Chapman *et al.* (3), with those found with diets containing 15% protein and 20% fat, the levels in the average diet. The procedure of Chapman *et al.* (3) gave a P.E.R. value of 3.25 for casein, whereas that using the higher levels of protein and fat gave a value of 3.06. The difference between these values, although slight, was statistically significant. On the other hand, both procedures gave comparable values for the plant protein mixture. These results indicate that there was little difference in the estimation of protein quality by the two methods. There is, however, a practical advantage of using the procedure of Chapman *et al.* (3), which is more applicable to low protein materials, such as cereal products. It may be impossible to test these foods at the 15% protein level without resorting to the dubious procedures of adding amino acids, concentrating the protein in the test material, or adding similar proteins.

The levels of carcass moisture, total lipid and protein, and of liver lipid found in experiment 1 were not significantly influenced by the diet fed (Table II). Analysis of the carcass gain showed that in animals fed casein diets, the increase in carcass protein per gram of protein consumed was uninfluenced by the dietary protein and/or fat level. In those fed plant protein, however, increasing

TABLE II

Effect of dietary protein and fat levels on carcass and liver composition and on efficiency of carcass protein synthesis

Diet	Dietary		Moisture, %	Carcass lipid, %	Protein, %	Liver lipid, %	Carcass protein synthesized, g/g protein intake
	Protein, %	Fat, %					
Casein	10	10	60.1±3.0*	18.0±3.7*	17.7±1.2*	7.0±1.3*	0.41
	15	10	61.5±2.2	17.0±3.4	18.1±1.0	6.2±1.8	0.37
	10	20	61.0±3.2	18.3±3.7	17.3±2.2	6.7±1.8	0.35
	15	20	60.2±3.3	18.8±4.2	17.8±1.5	6.4±1.5	0.42
Plant protein	10	10	63.7±2.9	15.4±2.8	16.7±1.3	6.9±2.8	0.07
	15	10	61.2±3.2	18.1±3.7	17.2±1.4	7.0±2.2	0.15
	10	20	62.8±2.3	14.9±2.7	17.2±1.2	6.7±1.8	0.05
	15	20	62.1±2.2	16.5±2.3	16.6±1.0	6.4±1.7	0.11

*Standard deviation.

the protein level increased the gain in carcass protein per gram of protein consumed, at both levels of fat tested. The efficiency of carcass protein synthesis was much lower in the animals given the plant protein diets than in those given the casein diets. Values found for carcass protein gained per gram of protein consumed did not correspond to P.E.R. values in all cases although the two measures of protein efficiency were related. For instance, increasing the protein level from 10 to 15% casein significantly reduced the P.E.R. from 3.25 to 2.75 in animals receiving 10% fat, but did not appreciably change the gain in carcass protein per gram of protein consumed. With both sources of protein, the efficiency of conversion of dietary protein to tissue protein was similar with diets containing 10% protein and 10% fat and those containing the levels of protein and fat in the average diet, 15% and 20% respectively. These results provide further evidence of the general validity of the procedure for P.E.R. given by Chapman *et al.* (3). There is no doubt, as Harris and Burress (5) have pointed out, that measurement of carcass protein synthesis is important in precise evaluations of protein quality. For practical regulatory or control purposes, however, it would appear that P.E.R. determinations, carried out under careful standardized conditions, will provide reliable estimates of the nutritional value of proteins.

Effect of Carbohydrate and Chlortetracycline

The results of experiment 2, conducted to study the effects on P.E.R. of carbohydrate source and the presence of an antibiotic in the diet, are summarized in Table III. It should be noted that the rats used in this study were

TABLE III

Effect of dietary carbohydrate source and 100 p.p.m. chlortetracycline on P.E.R. of casein

Carbohydrate source	Chlortetracycline added	Weight gain, g	Food consumption, g	P.E.R.
Cornstarch	–	67	227	2.93 ± 0.12*
	+	71	240	2.96 ± 0.15
Sucrose	–	61	206	2.96 ± 0.15
	+	66	221	2.97 ± 0.19

*Standard deviation.

obtained from a different source than those used in experiment 1 and hence the P.E.R. values obtained in the two experiments are not directly comparable. In agreement with the findings of Harper and Katayama (12), weight gains found with sucrose were less than those found with starch. Although Harper and Katayama reported that substitution of starch for sucrose spared the methionine requirement of rats, no difference in the P.E.R. of casein was found with the two carbohydrates in the present studies. A similar lack of effect of source of dietary carbohydrate on P.E.R. values of casein, wheat gluten, and soy grits was reported recently (13). The reason for the discrepancy between the present results and those of Harper and Katayama (12) is not apparent. It is not known whether P.E.R. values are a function of the dietary methionine level when

methionine is limiting, as in diets containing 10% casein, but it would appear reasonable to assume that if the substitution of starch for sucrose spared methionine, the difference between the carbohydrates should be manifested by a change in P.E.R. A relationship between net dietary protein value and total dietary sulphur has been demonstrated (**14**).

No effect of 100 p.p.m. chlortetracycline (Aureomycin) on P.E.R. values of casein found with either sucrose or cornstarch was observed, possibly because the antibiotic had no effect on weight gain.

References

1. P. H. Derse. J. Assoc. Offic. Agr. Chemists, **41**, 192 (1958).
2. J. A. Campbell and D. G. Chapman. J. Can. Diet. Assoc. **21**, 51 (1959).
3. D. G. Chapman, R. Castillo, and J. A. Campbell. Can. J. Biochem. and Physiol. **37**, 679 (1959).
4. A. B. Morrison and J. A. Campbell. J. Nutrition, **70**, 112 (1960).
5. R. S. Harris and D. A. Burress. J. Nutrition, **67**, 549 (1959).
6. Dietary Levels of Households in the United States. U.S. Dept. Agric., Washington, D.C. 1957.
7. Canadian Food and Nutrition Statistics, 1935 to 1956. The Queen's Printer, Ottawa. 1959.
8. J. Folch, M. Lees, and G. H. S. Stanley. J. Biol. Chem. **226**, 497 (1957).
9. G. W. Snedecor. Statistical methods. The Collegiate Press, Inc., Ames, Iowa. 1955.
10. T. B. Osborne, L. B. Mendel, and B. L. Ferry. J. Biol. Chem. **37**, 223 (1919).
11. R. G. Barnes, J. E. Maack, M. J. Knights, and G. O. Burr. Cereal Chem. **22**, 273 (1945).
12. A. E. Harper and M. C. Katayama. J. Nutrition, **49**, 261 (1953).
13. P. H. Derse. J. Assoc. Offic. Agr. Chemists, **43**, 38 (1960).
14. D. S. Miller and D. J. Naismith. Nature, **182**, 1786 (1958).

EFFECTS OF ANAEROBIOSIS ON THE RATES OF MULTIPLICATION OF MAMMALIAN CELLS CULTURED IN VITRO[1]

SAMUEL DALES[2]

Abstract

To test the effects of anaerobiosis on the rate of multiplication and carbohydrate metabolism of mammalian cells in vitro, cultures of a 'permanent' line, Earle's L strain cells, and of freshly explanted embryonic mouse cells were propagated in the presence and absence of oxygen. Contrary to the findings of several other investigators, our results show that the multiplication of both cell types was depressed by anaerobiosis. Anaerobiosis for at least 7 days, did not, however, bring about unbalanced growth in L cells, nor did it affect their capability to divide rapidly soon after they were returned to aerobic conditions. From the rates of glucose utilization, lactic acid production, and cell multiplication it was estimated that the rate of division in the two cell types studied was proportional to the energy which could be released from either glycolysis or complete oxidation of glucose.

Introduction

Primary cultures of embryonic cells and cells explanted from young animals have been reported to multiply equally well under aerobic and anaerobic conditions (9, 11, 12). In this respect Earle's L cells appeared to differ from these other cell types, for their growth was inhibited when the partial pressure of oxygen in the culture flasks was reduced below 0.1% (3). A number of possible reasons can be advanced to explain this difference. In previous experiments with L cells the cultures were maintained for several days without changing the nutrient medium and this resulted in an almost complete depletion of glucose in the medium of the anaerobic cultures. It was thus possible that division of L cells was being limited by a shortage of glucose rather than by the exclusion of oxygen. An alternative explanation might be that multiplication of L cells, which are a stable cell line, is much more sensitive to anaerobic conditions than is the multiplication of freshly isolated or embryonic cells. Finally it was also possible that the anaerobic conditions employed previously with L cells were more rigorous than those used in the studies on freshly isolated and embryonic cells.

To test these various possibilities, in the present investigation we have repeated our previous experiments with L cells (3) under more rigorous conditions, supplying glucose in abundant quantities through frequent changes of the nutrient medium. In an endeavor to obtain some further information about the role of glucose catabolism in providing energy for division of L cells under aerobic and anaerobic conditions, the rates of glucose consumption and production of lactic acid were determined and correlated with the rates of cell multiplication. In addition we have also studied the multiplication and carbohydrate metabolism of freshly explanted cells in the presence and absence

[1]Manuscript received January 25, 1960.
Contribution from the Ontario Cancer Institute, Toronto 5, Ontario. This work was done during the tenure of a Research Fellowship from the National Cancer Institute of Canada.
[2]Present address: Department of Cytology, The Rockefeller Institute, New York 21, N.Y.

of oxygen, using conditions of aerobiosis and anaerobiosis which were similar to those employed with L cells.

Materials and Methods

(a) Methods of Culturing Cells

Earle's L cells were propagated as suspensions of single cells in "roller-tubes" using the techniques of Siminovitch *et al.* (14). The individual cultures were kept in the same tubes throughout the experiment. To maintain the cell number in the cultures at $1–3 \times 10^5$ cells per milliliter, each day a fraction of the cell suspension was removed and the remaining cells were centrifuged down. After the old medium was poured off, the pellet of cells was resuspended in fresh nutrient medium and the cultures were returned to the incubator.

Cultures of Swiss mouse embryo cells were prepared from nearly full-term foetuses (about 20 days old). Several embryos were removed from the uterus and were cut into small pieces. The tissue, suspended in PBS (Dulbecco and Vogt's balanced saline (5)), with 0.25% trypsin added, was agitated for 1 hour at 37° C in a 250-ml Erlenmeyer flask, by means of a magnetic stirrer. The resulting suspension of cells was decanted, leaving large pieces of tissue behind, and was pipetted into culture bottles containing nutrient medium. After 24 hours, when the viable cells had settled on the glass surface and begun to multiply, the medium and cell debris were removed and the cells were washed with PBS. They were then removed from the glass with the PBS–trypsin solution. Accurately measured aliquots of this cell suspension were pipetted into 250-ml culture bottles and fresh medium was added. Following another period of 24 hours the medium was once more replaced and the flasks were gassed. Cells were removed from the glass surface for counting by the trypsinization procedure described above. The ratio of liquid to gas volume in the flasks was 1:20.

The nutrient medium used to culture both L cells and embryonic mouse cells was CMRL-1066 (modified medium 858 (10), as sold commercially by the Connaught Medical Research Laboratories, University of Toronto) supplemented with 20% horse serum. An effort was made during handling to keep the cultures at 37° C so as to prevent a slowing down of cell multiplication which can result from cooling.

(b) Gassing Procedures

In order to make the cultures anaerobic and at the same time maintain sterility in the medium, two short lengths of glass tubing were pushed through each silicone stopper and acted as an inlet and outlet for the gases. Pressure tubing was attached to the outer end of the glass tubes and onto the ends of the pressure tubing were attached glass bulbs stuffed with cotton plugs. To free the cultures of oxygen and to adjust the pH of the medium to approximately 7.2, a mixture consisting of about 96% of the purest commercially available nitrogen (upper limit of oxygen less than 0.075%) and of about 4% carbon dioxide was passed over the surface of the cell suspension at the rate of 1 liter per minute for 15 minutes. The liquid in the culture flasks was agitated throughout the period of gassing to facilitate gas exchange. In the

cultures which were kept anaerobic for several days air was admitted for a period of 10–15 minutes each day when the nutrient medium was being changed. In the controls air mixed with carbon dioxide (bubbled into the medium to adjust the pH to about 7.2) constituted the gas phase.

The cells in suspension were enumerated in a Spencer hemacytometer. Glucose in the nutrient medium was analyzed by the method of Somogyi (15) and lactic acid was analyzed by the procedure of Barker and Summerson, modified by Umbreit and others (16).

Results

Multiplication of L Cells and Mouse Embryonic Cells under Aerobic and Anaerobic Conditions

The results of a typical experiment comparing the multiplication of L cells in the presence and absence of oxygen are shown in Fig. 1. Each point is the average cell number in two replicate cultures, which were sampled daily. It is

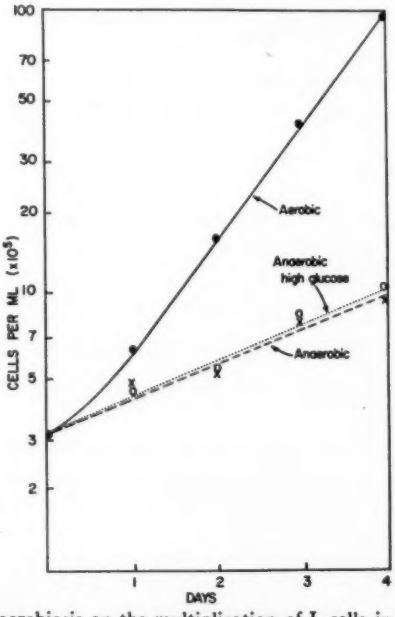


FIG. 1. Effect of anaerobiosis on the multiplication of L cells in suspension. The high glucose medium contained three times the normal amounts of glucose.

evident that in the presence of oxygen, cells multiplied at a faster rate than in its absence. Thus the time required for the cell number to double was 19 hours in the aerobic and 60 hours in the anaerobic cultures. It is also clear from the results shown in Fig. 1, that glucose was not the factor limiting cell division under anaerobic conditions since the addition of glucose at higher concentrations (3 mg per ml instead of 1 mg per ml) did not accelerate the slow rate of multiplication in the anaerobic cultures.

Similar experiments to those carried out with L cells were performed using mouse embryonic cells in stationary cultures, in vitro. The results of a representative experiment on the rate of multiplication in the presence and absence of oxygen are shown in Fig. 2. Each point plotted is the average of counts made on three replicate cultures. Evidently the growth of these 'normal' cells was also reduced by anaerobiosis and the time required to double their number increased from 30 to 68 hours.

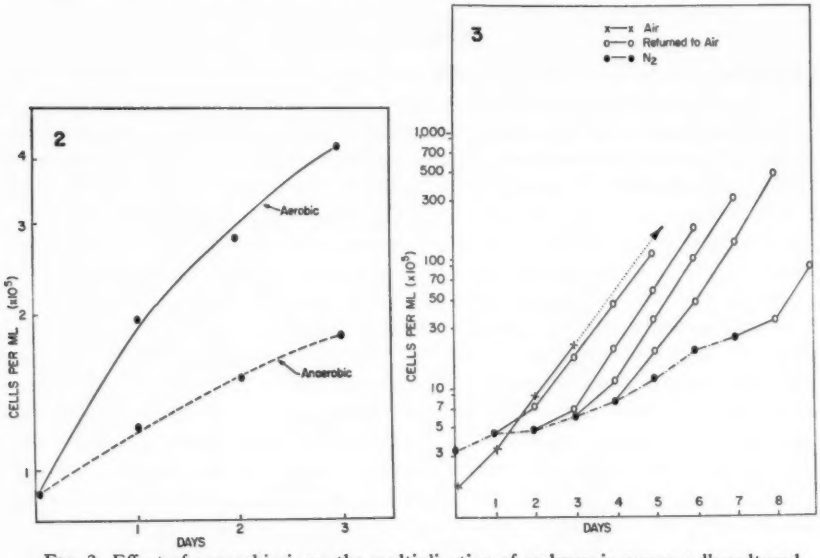


FIG. 2. Effect of anaerobiosis on the multiplication of embryonic mouse cells cultured on glass.

FIG. 3. Effect of aerobiosis, following various periods of anaerobiosis, upon the multiplication of L cells in suspension.

To test whether the capability of L cells to multiply rapidly had been affected by anaerobiosis, samples of cells were removed from the anaerobic cultures on 4 successive days and on the 7th day and thereafter were cultured under aerobic conditions. The results of a representative experiment, in which the average cell numbers in two replicate cultures are represented by each point plotted, are shown in Fig. 3. Again multiplication was much slower when oxygen was absent. On being returned from anaerobic to aerobic conditions the cells resumed the rate of multiplication associated with aerobic cultures. Clearly, anaerobiosis for even as long as 7 days had no adverse effect on the capability of L cells to multiply rapidly in oxygen-containing medium.

Carbohydrate Metabolism of L *Cells and Mouse Embryonic Cells Growing under Aerobic and Anaerobic Conditions*

In addition to estimating the daily increase in cell populations we also determined the amounts of glucose utilized and lactic acid produced. With

these data it is possible to calculate the glucose utilized and lactic acid produced in a given period by an exponentially increasing population of cells by writing the equation

$$\int_0^C dc = kN_0 \int_0^T e^{\alpha t}\, dt$$

where C is the total glucose taken up or lactic acid produced per milliliter of medium in time T, which is the time interval between determinations (24 hours), k is the rate per cell per hour, N_0 is the initial number of cells, and N the final number at the end of each 24-hour period, $\alpha = 0.693/G$ where G is the mean generation time, dc/dt is the rate of glucose uptake or lactic acid production per cell at any instant.
This integration simplifies to

$$k = \frac{C}{T}\left[\frac{\ln N - \ln N_0}{N - N_0}\right].$$

The means of k values, accompanied by their standard errors, for glucose utilization and lactic acid production by L cells and mouse embryonic cells are given in Tables I and II respectively.

TABLE I

Glucose consumption and lactic acid production by L cells

The gas phase (*a*)		μg glucose used/hr/cell (*b*)		μg lactic acid produced/hr/cell (*c*)	
Air/CO_2	(1)	$2.2\times10^{-5}\pm0.4\times10^{-5}$	(6)*	$4.5\times10^{-6}\pm0.5\times10^{-6}$	(6)*
N_2/CO_2	(2)	$7.2\times10^{-5}\pm0.5\times10^{-5}$	(9)*	$4.6\times10^{-5}\pm0.2\times10^{-5}$	(9)*
N_2/CO_2 high glucose medium	(3)	$14.0\times10^{-5}\pm0.4\times10^{-5}$	(4)*	$5.4\times10^{-5}\pm0.3\times10^{-5}$	(4)*
Returned from N_2/CO_2 to air/CO_2	(4)	$3.1\times10^{-5}\pm0.4\times10^{-5}$	(18)*	$5.2\times10^{-6}\pm0.8\times10^{-6}$	(18)*

*The number of determinations on replicate samples of medium from cultures in three or four different experiments are shown in parentheses following the average k value and its standard error.

Comparing lines (1) and (2) of column (*b*) in Table I, it can be seen that glucose uptake by L cells was almost 3 times greater under anaerobic conditions. When additional glucose was present in the nutrient medium the anaerobic stimulation of glucose consumption was even greater (line (3) of column (*b*)). Of the glucose consumed by L cells in the presence of oxygen only about 20% could be detected as lactic acid appearing in the medium. In contrast to this relatively low rate of aerobic glycolysis, the anaerobic glycolysis accounted for about two-thirds of the glucose consumed (compare lines (1) and (2) of column (*c*) in Table I). The very high consumption of glucose in the high glucose medium was not, however, accompanied by a proportional increase in the production of lactic acid (line (3), columns (*b*) and (*c*)), for, in fact, only about the same amount of lactate was produced anaerobically in

the normal and high glucose medium. This observation suggests that the extra glucose taken up from the high glucose medium did not become converted into lactic acid because the glycolytic reactions in L cells were already functioning at maximal rates.

Results of analyses made on the medium of cultures in which L cells were growing after being returned from anaerobic to aerobic conditions are given in line (4) of Table I. From these it may be seen that the resumption of rapid growth, illustrated in Fig. 2, resulted also in a return of the rates of glucose consumption and lactic acid production to the levels normally found in aerobic cultures.

The results of analyses made on the nutrient medium in which embryonic mouse cells had been propagated are shown in Table II. From columns (*b*) and (*c*) it may be seen that the rate of glucose consumption by these cells was

TABLE II

Glucose consumption and lactic acid production by mouse embryonic cells

The gas phase (*a*)	μg glucose used/hr/cell (*b*)	μg lactic acid produced/hr/cell (*c*)
Air/CO_2 (1)	$4.2\times10^{-5}\pm0.9\times10^{-5}$ (6)*	$3.2\times10^{-5}\pm0.2\times10^{-5}$ (6)*
N_2/CO_2 (2)	$11.0\times10^{-5}\pm0.5\times10^{-5}$ (5)*	$9.5\times10^{-5}\pm0.9\times10^{-5}$ (5)*

*The number of determinations on pooled samples of medium from three replicate cultures, collected on consecutive days in two different experiments, are shown in parentheses following each average *k* value and its standard error.

of the same order as that of L cells, but the amount of glucose converted into lactic acid in the aerobic cultures (about 75% of the glucose consumed) was much greater than that converted by L cells. As was the case with L cells, anaerobiosis stimulated glucose uptake but here most of this (86%) was converted into lactic acid.

Discussion

The results of the present experiments show that the presence of oxygen is necessary for the maintenance of cell division at a maximum rate by a permanent line of mouse cells, the L strain, and also by freshly isolated mouse embryonic cells. These findings are, therefore, in agreement with our previous results on L cells, but are at variance with the results obtained by others who studied the in vitro growth of vertebrate cells under anaerobic conditions. Thus Harris (9) in his studies on the multiplication of rat connective tissue, Jones and Bonting (11) in their experiments on the growth of explanted chick embryonic lung and intestine, and Pomerat and Willmer (12), who worked with chick embryo fibroblasts, all reported equally good growth under aerobic and anaerobic conditions. These observations suggest that the ability of cells in vitro to multiply rapidly under anaerobic conditions may be determined by their origin. However, the information now available regarding the role of carbohydrate metabolism in providing the energy for growth of vertebrate cells suggests that since much less energy is produced by glycolysis than by aerobic oxidation it is to be expected that a reduction of growth will occur

when oxygen is removed from the environment of growing cells (1). If we assume that the energy for growth is made available in the form of 'high-energy' bonds of the ATP molecule and that approximately 12 times more energy is released by aerobic oxidation than by glycolysis (7), then it can be calculated from the data on the rates of glucose utilization and lactic acid production, given in Table I, that production of energy by L cells in the aerobic cultures is about 3.7 times greater than in the anaerobic ones. Similarly from the data in Table II it can be calculated that in mouse embryonic cells the energy produced under aerobic conditions is 2.5 times greater than that produced under anaerobic ones. Using the time required to double the cell concentration we can say that in the presence of oxygen L cells grew 3.2 times faster and mouse embryonic cells 2.3 times faster than in its absence. Essentially similar results have been obtained with another permanent line of cells, the strain HeLa of human cancer origin, which has a rate of glycolysis even greater than that of the embryonic mouse cells (4), thus indicating that in these cells also aerobic oxidation is concerned with promoting energy for the most rapid rate of division. The failure of increased amounts of glucose to stimulate either the anaerobic production of lactic acid or the division of L cells indicates further that the glycolytic pathways, already functioning maximally at normal glucose concentrations, are unable to furnish additional energy for division. These results taken together emphasize once more the direct relationship between energy production from carbohydrate breakdown and division of mammalian cells. It is, therefore, surprising that Harris (9) failed to observe a reduction of cell division in anaerobic cultures of the rat heart cells used by him, since our calculations, based on the data presented by Harris, show that the rates of glucose utilization, aerobic glycolysis, and anaerobic glycolysis are identical in the cells used by him and in the embryonic mouse cells employed in the present experiments.

The resumption of rapid growth upon the return of L cells from anaerobic to aerobic conditions indicates that a prolonged removal of oxygen from their environment fails to affect the capacity of these cells to proliferate rapidly. In this respect L cells behave somewhat like *Escherichia coli*, which were found by Fowler (6) to grow exponentially without any lag when these anaerobically cultured bacteria were returned to aerobic conditions. Unlike the L strain cells these bacteria were, however, able to resume rapid growth under anaerobic conditions following a short lag period of adaptation. Since in anaerobic cultures L cells remain unchanged in their size and their RNA and DNA content (4), it must be assumed that no 'imbalanced' growth occurs under these conditions. This is in contrast with the effects of respiratory inhibitors, such as the narcotic luminal, which not only inhibit cell division but also bring about a formation of giant cells which have an increased content of nitrogen and DNA (2). It appears, therefore, unlikely that in anaerobic cultures of L cells the slow rate of division in the culture as a whole results from a rapid division of a small proportion of the cells and no division by the remainder, but rather that all the cells are equally affected and grow at a slow rate.

Experiments are being carried out at the present time by numerous investigators, using mammalian cell cultures, which deal with such problems as the relationship between viruses and host cells, with the turnover of cellular materials and with the effects of hormones on cell metabolism. Thus, for example, in experiments reported recently by Graff *et al.* (8) the effect of insulin upon the metabolism of L cells was investigated. The L cells used by Graff *et al.* were dividing at a relatively slow rate and glycolyzing at a very high rate, much as they do under conditions of oxygen deprivation or in the presence of inhibitors. In order to make a complete evaluation of the results of experiments in which cultured cells are used it might be useful to know what is the metabolic state of such cells in relation to the optimal condition which can be attained. Such an evaluation can, in part, be obtained by experiments such as those reported here, in which the rates of division under optimal and less optimal conditions are compared.

Acknowledgments

I am greatly indebted to Dr. L. Siminovitch for his guidance and interest throughout. I also wish to thank Drs. C. W. Helleiner, J. E. Till, C. R. Fuerst, and Mr. F. Kahan for their helpful advice. The formula used to calculate the rates of glucose breakdown was kindly suggested to me by Mr. F. Kahan.

References

1. W. S. Bullough. Biol. Revs. **27**, 133–168 (1952).
2. S. Dales and K. C. Fisher. J. Cellular Comp. Physiol. **51**, 359–384 (1958).
3. S. Dales and K. C. Fisher. Can. J. Biochem. and Physiol. **37**, 623–638 (1959).
4. S. Dales. Unpublished data.
5. R. Dulbecco and M. Vogt. J. Exptl. Med. **99**, 167–182 (1954).
6. C. Fowler. Biochim. et Biophys. Acta, **7**, 563–573 (1951).
7. J. S. Fruton and S. Simmonds. *In* General biochemistry. 2nd ed. John Wiley & Sons, Inc., New York. 1958. pp. 520–524.
8. S. Graff and K. S. McCarty. Cancer Research, **18**, 741–746 (1958).
9. H. Harris. Brit. J. Exptl. Pathol. **37**, 512–517 (1956).
10. G. M. Healy, D. C. Fisher, and R. C. Parker. Proc. Soc. Exptl. Biol. Med. **89**, 71–77 (1955).
11. M. Jones and S. L. Bonting. Exptl. Cell Research, **10**, 631–639 (1956).
12. C. M. Pomerat and E. N. Willmer. J. Exptl. Biol. **16**, 232–249 (1939).
13. K. K. Sanford, W. R. Earle, and G. D. Likely. J. Natl. Cancer Inst. **9**, 229–246 (1948).
14. L. Siminovitch, A. F. Graham, S. M. Lesley, and A. Nevill. Exptl. Cell Research, **12**, 299–308 (1957).
15. M. Somogyi. J. Biol. Chem. **160**, 69–73 (1945).
16. W. W. Umbreit, R. H. Burris, and J. F. Stauffer. Manometric techniques and tissue metabolism. 2nd ed. Burgess Publishing Co., Minneapolis, Minn. 1949. p. 192.

DIETARY MARINE FISH OILS AND CHOLESTEROL METABOLISM

2. THE EFFECT OF VITAMIN A AND LINGCOD LIVER OIL COMPONENTS ON THE SERUM CHOLESTEROL LEVELS IN CHICKS[1]

J. D. Wood

Abstract

Lingcod liver oil unsaponifiable material was separated into three main fractions by means of an aluminum oxide column. Major components of the three fractions were vitamin A, cholesterol, and glyceryl ethers, respectively. These fractions were given as dietary supplements to cholesterol-fed chicks and the effect of the supplements on the hypercholesterolemia induced by the cholesterol feeding was investigated. The fraction containing vitamin A prevented the hypercholesterolemia. Crystalline vitamin A acetate produced a similar effect when it was added as a dietary supplement. It was concluded that vitamin A was probably the hypocholesterolemic agent in lingcod liver oil although other compounds in the oil may also exert some influence on the control of the serum cholesterol concentrations in the chicks.

Introduction

Numerous reports have appeared in the literature in recent years on the effect of dietary fats and oils on cholesterol levels in the blood. These studies have included several which indicated that dietary fish oils can depress serum cholesterol levels in both man (1, 2, 3) and animals (4). The effectiveness of various marine fish oils in preventing hypercholesterolemia in cholesterol-fed chicks was studied by Wood and Biely (5), who found that lingcod liver oil and halibut liver oil were very efficient hypocholesterolemic agents. Further work in this laboratory (6) showed that the active factor in lingcod liver oil was present in the unsaponifiable fraction of the oil. This result was the opposite to that observed by De Groot and Reed (4) using rats. These workers found that cod liver oil depressed the serum cholesterol concentrations but that the depressant factor was present in the fatty acid fraction. The different effects observed with chicks and rats may be due to a species difference, or to the different composition of the fish oils employed in the investigations.

The liver oils of the lingcod and the halibut were most effective in preventing hypercholesterolemia in chicks as noted above. Moreover, these oils have a high content of vitamin A, being used commercially as a source of this vitamin. The possibility that vitamin A was the active factor in lingcod liver oil therefore appeared to be worth investigating. Experiments were carried out in an attempt to identify the hypocholesterolemic factor in lingcod liver oil unsaponifiable material and at the same time to study the influence of vitamin A on serum cholesterol levels in cholesterol-fed chicks.

Materials and Methods

Birds

New Hampshire chicks were used in the earlier experiments but more recently White Leghorn chicks were employed because they were more readily available.

[1]Manuscript received April 12, 1960.
Contribution from the Fisheries Research Board of Canada, Technological Station, Vancouver 8, B.C. Part 1 of this series was published in the Canadian Journal of Biochemistry and Physiology, **38**, 19 (1960).

Lingcod liver oil was found to have similar effects on serum cholesterol levels in both types of birds. One-week-old cockerels were used in the experiments and they were standardized by weight to eliminate any possibility of variation in serum cholesterol levels due to differences in the size of the chicks.

Diets

The basal diet employed in the experiments was the same as that used previously (5). Cholesterol and the various dietary supplements were added in amounts detailed in the text and tables.

Determination of Serum Cholesterol

The birds were placed in groups of 16 and fed the appropriate diet and water ad libitum. After the required period of time the birds were bled from the wing and 0.5 ml blood collected from each chick. The blood from four birds was pooled and the serum collected in the normal manner. There were therefore four pooled samples of serum from birds on each of the diets. The free and total cholesterol contents of the samples were determined using the method of Sperry and Webb (7).

Preparation of Lingcod Liver Oil Unsaponifiable Material

One pound of lingcod liver oil was mixed thoroughly with 4 liters ethanol and 300 ml of a solution ($KOH:H_2O$, 3:2 w/v). The mixture was boiled gently under reflux for 30 minutes, during which time it was protected from light. The mixture was distilled until 2 liters ethanol had been removed and the remaining solution, after dilution with 4 liters water, was extracted several times with ethyl ether to obtain the unsaponifiable material. The above proportions of alcohol and water were chosen because they reduced to a minimum the formation of emulsions during the extractions. The ether extracts were combined and washed successively with water, dilute potassium hydroxide, and then with water until the washings were no longer alkaline to phenolphthalein. The solvent was removed on a water bath at 60° C under a stream of nitrogen and finally *in vacuo* at room temperature.

Preparation of Lingcod Liver Oil Fatty Acids

The saponification mixture, after removal of the unsaponifiable material, was acidified with hydrochloric acid and extracted several times with ethyl ether and the combined extracts washed with water. The solvent was removed as described above.

Fractionation of Lingcod Liver Oil Unsaponifiable Material

The fractionation was based on the method of Swain (8) and was carried out using a 75-mm diameter column containing 900 g of aluminum oxide (Woelm, basic, activity grade 1) equilibrated with 5% water. A slurry of the adsorbent in petroleum ether (b.p. 65–110°) was poured into the column which was fitted at the bottom with a perforated, fritted glass disk plus a thin layer of glass wool. The unsaponifiable material under investigation was dissolved in petroleum ether to give a final concentration of 2%. This solution was applied to the column followed by more petroleum ether, benzene, methylene chloride, and methanol in that order. The effluent was collected in fractions varying in

volume from 100 ml to 2 liters, the volume depending on the type and amount of material being eluted at that specific moment. The volumes of the fractions were chosen in the light of results obtained in previous runs and were smallest when the vitamin A and cholesterol were being eluted and largest towards the completion of the elution when nearly all the material had already been eluted.

In preliminary runs using aluminum oxide columns the benzene was omitted, the eluting solvent being changed directly from petroleum ether to methylene chloride. However, vitamin A and cholesterol were not separated very well by this method and it was found that the use of benzene as an intermediate solvent increased the efficiency of this separation. It was therefore used in the fractionation of the unsaponifiable material in the present investigation. The volumes of the individual solvents used are shown in Fig. 1.

Results

The chromatographic separation of the components of lingcod liver oil unsaponifiable matter is shown in Fig. 1. The material adsorbed on the aluminum oxide column was eluted in four major fractions. The first occurred with the benzene as solvent, the second and third with methylene chloride as solvent, and the fourth when the elution was carried out with methanol. The petroleum ether eluted only a trace of material. The recovery of the material from the column was excellent, 43.2 g of the original 43.6 g applied to the column being recovered in the eluate.

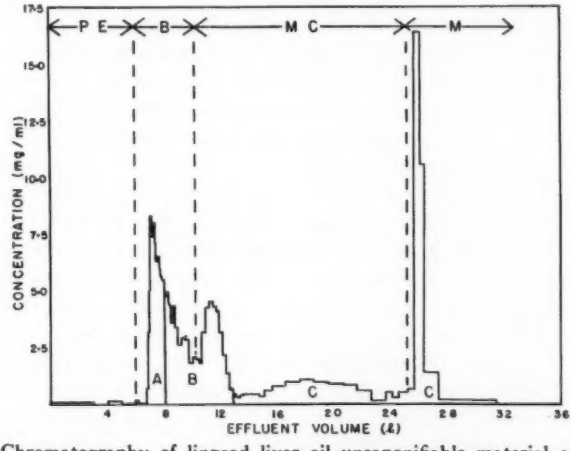


FIG. 1. Chromatography of lingcod liver oil unsaponifiable material on aluminum oxide. Solvents were petroleum ether b.p. 65°–110° (PE), benzene (B), methylene chloride (MC), and methanol (M).

The first peak in Fig. 1 contained large amounts of vitamin A which was identified by its ultraviolet absorption spectra and by the absorption spectra of its reaction product with antimony trichloride. The material under investigation showed maxima at 328 mμ and 620 mμ in these two tests, respectively. An accurate determination of the amount of vitamin A in the fractions was not

possible because of interfering substances, but the results obtained indicated that there was a considerable quantity of material present in the first peak which was neither vitamin A nor cholesterol. The semiquantitative estimation of vitamin A indicated that an appreciable amount of the vitamin had been destroyed during its isolation from the oil, possibly while the material was adsorbed on the column. The Liebermann–Burchard test for cholesterol indicated that the sterol was present in significant amounts in the fractions which made up the latter part of the first peak in the diagram. Vitamin A was also present in these fractions but not to the same extent as in the earlier fractions of the peak.

The second peak in Fig. 1 represents the material eluted immediately after the solvent was changed from benzene to methylene chloride. This material was solid and pale yellow in color and was almost entirely cholesterol as estimated by the Liebermann–Burchard reagent. This reagent is not specific for cholesterol and two further tests were carried out to identify the sterol. The time development of the color with the Liebermann–Burchard reagent was followed (9) and the development of the color was found to be identical with that obtained using a cholesterol standard. Further proof that the material was cholesterol was obtained from the melting point. The pale yellow solid melted at 141.5° C but recrystallization from 95% ethanol gave a white crystalline solid, m.p. 148° C. Further recrystallization had no effect on the melting point, which was identical with that observed with a sample of pure cholesterol. The mixed melting point of the two substances was also 148° C.

The compounds represented by the third and fourth peaks in Fig. 1 contained no traces of either vitamin A or cholesterol and their physical states were an oil and a waxy solid respectively. The glyceryl ether content of the two fractions was obtained, using the method of Karnovsky and Rapson (10). The amount of glyceryl ethers was calculated (as selachyl alcohol) from the amount of formaldehyde produced during periodate oxidation. Formaldehyde was estimated using the dimedon reagent and the identity of the aldehyde was checked by taking the melting point of the dimedon precipitate. The value obtained was 189°, which agreed with the value for formaldehyde–dimedon compound observed by Reeves (11). The materials represented by peaks 3 and 4 were found to contain 59.7 and 96.5% glyceryl ethers respectively.

The components of the unsaponifiable material, which were thus separated, were tested for their effectiveness in preventing hypercholesterolemia in cholesterol-fed White Leghorn chicks. The material was divided into three fractions, A, B, and C, as shown in Fig. 1. The limits of fraction A were chosen so that it contained most of the vitamin A and none of the cholesterol. Fraction B contained the material represented by the second peak in Fig. 1, plus the latter fractions making up the first peak since these fractions contained substantial amounts of cholesterol in addition to some vitamin A. Fraction B therefore consisted mainly of cholesterol with a little vitamin A. The material represented by the third and fourth peaks was combined to form fraction C, which contained mainly glyceryl ethers but also some unidentified compound or compounds.

TABLE I

The effect of dietary vitamin A and lingcod liver oil components on the serum cholesterol levels in chicks

Addition to the basal diet	Food consumption (g/chick/day)		Average weight gain (g)		Serum cholesterol (mg/100 ml) Free		Serum cholesterol (mg/100 ml) Total		Cholesterol in free state (%)	
	7 days	13 days	7 days	13 days	7 days	13 days	7 days	13 days	7 days	13 days
None	17.3	19.5	43	91	38 ± 1	36 ± 2	137 ± 13	115 ± 8	28 ± 4	31 ± 4
1% cholesterol (Ch)	16.4	19.7	41	93	84 ± 17	101 ± 16	289 ± 37	336 ± 29	28 ± 3	31 ± 5
Ch + 10% lingcod liver oil	17.3	19.3	42	77	26 ± 4	31 ± 5	116 ± 11	91 ± 12	23 ± 4	34 ± 1
Ch + fraction A*	15.1	20.0	41	89	58 ± 6	61 ± 3	218 ± 25	197 ± 28	27 ± 1	31 ± 3
Ch + fraction B*	17.3	20.7	43	92	84 ± 24	79 ± 5	285 ± 37	248 ± 46	29 ± 4	32 ± 6
Ch + fraction C*	16.7	19.6	39	85	88 ± 17	126 ± 30	271 ± 59	429 ± 119	33 ± 1	30 ± 3
Ch + 580,000 I.U. vitamin A acetate/lb	16.4	20.4	39	87	42 ± 4	44 ± 5	146 ± 20	163 ± 12	30 ± 2	27 ± 2
Ch + 290,000 I.U. vitamin A acetate/lb	16.2	19.0	40	86	54 ± 8	56 ± 12	180 ± 21	190 ± 18	30 ± 7	30 ± 9
Ch + 145,000 I.U. vitamin A acetate/lb	18.4	20.6	42	88	44 ± 6	48 ± 6	159 ± 27	141 ± 15	28 ± 1	34 ± 1
Ch + 72,500 I.U. vitamin A acetate/lb	17.1	19.7	43	89	48 ± 5	62 ± 14	166 ± 24	175 ± 12	29 ± 5	35 ± 6
Ch + 36,250 I.U. vitamin A acetate/lb	18.1	19.9	41	83	58 ± 8	77 ± 12	217 ± 38	240 ± 36	27 ± 1	32 ± 1
Ch + 18,125 I.U. vitamin A acetate/lb	17.0	20.2	42	88	57 ± 6	65 ± 7	217 ± 24	196 ± 41	26 ± 1	33 ± 9
Ch + 9,062 I.U. vitamin A acetate/lb	16.7	21.1	43	90	53 ± 2	66 ± 19	201 ± 14	196 ± 39	27 ± 2	34 ± 4
Ch + 330,000 I.U. vitamin A alcohol/lb	18.0	20.7	45	89	52 ± 15	69 ± 14	195 ± 47	234 ± 45	26 ± 2	29 ± 2

*Fractions A, B, and C were added to 10 lb of the basal diet.

NOTE: Each cholesterol value in the table is the mean for four groups ± the standard deviation.

The effect of supplementing the diet of cholesterol-fed chicks with fractions A, B, and C and lingcod liver oil are shown in Table I. The food consumption of the chicks and their weight gain were similar for birds on all the diets after both 7 days and 13 days except for those on the lingcod liver oil supplemented diet. After 13 days on the latter diet the birds had consumed the normal amount of food but their weight gain was somewhat less than that of birds on the other diets.

The influence of the different dietary supplements on the prevention of hypercholesterolemia in the chicks was varied. After 7 days the chicks fed diets supplemented with fraction A or lingcod liver oil exhibited serum cholesterol levels which were lower than the levels in the cholesterol-fed control birds. Analysis of variance indicated that the differences were significant at $P = .01$. The same level of significance is used in all cases in this paper where a significant difference is mentioned. Fraction A and lingcod liver oil produced similar effects after 14 days on the diet. Fraction B had no effect on the serum cholesterol levels in cholesterol-fed chicks after 7 days on the diet but at the end of 13 days a significant lowering of the cholesterol concentration was observed. Fraction C produced no effect after 7 days but after 13 days on the diet the cholesterol levels were significantly higher than the cholesterol-fed controls. A group of chicks was placed on the basal diet without any added cholesterol and it was observed that lingcod liver oil lowered the serum cholesterol concentration in cholesterol-fed chicks to a value below that in the chicks on the basal diet. The other fractions capable of reducing the rise in serum cholesterol did not produce such a strikingly low level of cholesterol.

The alterations in the serum cholesterol levels brought about by the dietary supplements applied both to the free cholesterol and to the total amount of the compound. Dietary supplements which drastically changed the levels of serum total cholesterol had no effect on the percentage of cholesterol in the free state. The results in Table I show that this percentage composition varied little, not only between birds on the same diet, but also between birds on different diets.

The ability of vitamin A to prevent hypercholesterolemia in cholesterol-fed chicks is shown in Table I. Both the alcohol and acetate form of the vitamin significantly lowered the serum cholesterol concentration. Although the hypocholesterolemic effect of the vitamin A acetate increased somewhat with concentration, the increase was rather erratic and it appeared that vitamin A concentration was not too critical at concentrations greater than 9062 I.U./lb feed. The values of vitamin A shown in Table I indicate the amount of the crystalline vitamin added to the diets. All diets, in addition to this supplement, contained 2500 I.U./lb vitamin A derived from feeding oil used to formulate the basal diet. None of the vitamin A supplemented diets lowered the serum cholesterol levels to the same extent as did the lingcod liver oil supplemented diet. The fish oil was rich in vitamin A (42,500 I.U./g), which would result in an addition of 1,929,500 I.U. of vitamin A per pound of feed. This level is approximately three times greater than the highest level of crystalline vitamin A added to the diets. The action of the dietary vitamin A was similar to that of the fish oil and its components in that it had no effect on the proportions of cholesterol

in the free and esterified states, respectively. The amounts of the basal diet and vitamin A supplemented diets consumed by the chicks were similar. In this respect chicks differ from rats, which were found to eat less of the vitamin A supplemented diets (unpublished results).

As mentioned previously, the unsaponifiable portion of lingcod liver oil contained the hypocholesterolemic factor whereas the fatty acid portion obtained after the saponification of the oil increased the degree of hypercholesterolemia in cholesterol-fed chicks (6). The effect of the unsaponifiable material on the hypercholesterolemia caused by the fatty acids was therefore studied because the results of such an experiment might shed some light on the mechanism involved in the control of serum cholesterol levels by dietary means.

Lingcod liver oil was saponified and the unsaponifiable material and free fatty acid fractions were isolated as described above. The whole oil and the two fractions (singly and combined) were added to the diets of New Hampshire chicks as shown in Table II. The amount of unsaponifiable material which

TABLE II

The effect of dietary lingcod liver oil and its components on the serum cholesterol level in chicks

Addition to the basal diet	Serum cholesterol (mg/100 ml), 10 days
None	119 ± 5
1% cholesterol	258 ± 51
1% cholesterol + 10% oil	83 ± 9
1% cholesterol + 300 g fatty acid/10 lb feed	315 ± 58
1% cholesterol + unsaponifiable material	110 ± 18
1% cholesterol + 300 g fatty acids + unsaponifiable material	137 ± 16

NOTE: Each value in the table is the mean for four groups ± the standard deviation.

was used to supplement the diets was chosen so that it was equivalent to that obtained from the quantity of oil used as the dietary supplement. In agreement with previous results the oil and unsaponifiable material prevented the induced hypercholesterolemia and the free fatty acids enhanced it. The unsaponifiable material was able to counteract the effect of the fatty acids when the two fractions were both added to the diet. The whole oil and the unsaponifiable portion not only prevented the induced hypercholesterolemia but in addition they produced serum cholesterol levels which were lower than those of the chicks on the basal diet.

Discussion

The results observed when the components of lingcod liver oil unsaponifiable material were added as dietary supplements to cholesterol-fed chicks would indicate that vitamin A may be the hypocholesterolemic factor in the fish oil. Of the fractions tested, that containing the main portion of vitamin A was the most effective in preventing the rise in serum cholesterol induced by dietary cholesterol. The fraction containing a small amount of the vitamin produced a significant effect only after 13 days on the diets, there being no significant

prevention of the hypercholesterolemia after 7 days. The fraction which was devoid of vitamin A increased the cholesterol-induced hypercholesterolemia. The activity of the isolated vitamin A was not directly comparable with that of the oil because a certain amount of the vitamin had been destroyed during the isolation and the remainder was not contained wholly in any one fraction.

The results obtained with crystalline vitamin A acetate would appear to substantiate the hypothesis that vitamin A is the active factor in lingcod liver oil. The crystalline vitamin at all levels investigated, 9062 I.U./lb feed to 580,000 I.U./lb feed, curtailed the rise in serum cholesterol due to dietary cholesterol.

However, the effect of crystalline vitamin A acetate was not so great as that of the lingcod liver oil, nor for that matter the unsaponifiable fraction of the oil. The latter two substances when added to a cholesterol-containing diet produced serum cholesterol levels which were lower than those found in the chicks on the basal diet without added cholesterol. The greater potency of the fish oil may be due to its high content of vitamin A. The amount of the vitamin added to the diet in the oil was three times as much as the highest concentration of crystalline vitamin A tested. However, the rather indefinite increase in the hypocholesterolemic effect with increase in the vitamin A concentration would seem to make this improbable. Moreover, lingcod liver oil with a lower vitamin A content was used in a previous experiment (5). In this case the oil-supplemented diet contained 900,000 I.U./lb feed, which is of the same order of concentration as the 580,000 I.U./lb feed used here, and yet the oil prevented completely the induced hypercholesterolemia as compared with the partial prevention effected by the crystalline vitamin. The possibility therefore exists that there is some compound or compounds apart from vitamin A which are present in lingcod liver oil and which play some role in the regulation of serum cholesterol levels. This unknown substance may itself have hypocholesterolemic properties or it may stimulate the activity of the vitamin A. Take, for example, the glyceryl ethers which are present in appreciable quantities in lingcod liver oil. They produce no lowering of serum cholesterol concentrations per se but they are known to act as emulsifying agents. The potent hypocholesterolemic activity of fish liver oil may be due to the ability of the glyceryl ethers to increase the degree of dispersion of vitamin A in aqueous media. These suggestions are of course speculative and studies are now being planned to investigate this aspect more thoroughly.

The effect of vitamin A on blood cholesterol levels in poultry has been studied previously by Weitzel *et al.* (12). These workers gave large oral doses of vitamin A to old atherosclerotic hens, and although a marked anti-atherosclerotic effect was observed, the changes in the serum cholesterol were slight. These results are contrary to the present findings but Weitzel and his co-workers were studying the lowering of cholesterol levels as distinct from the present study, which was devoted to the prevention of a rise in serum cholesterol concentration due to dietary cholesterol. In addition, the cholesterol metabolism may be quite different in young cockerels and old hens. The two investigations are not therefore comparable.

The present investigations give little or no indication as to the site of the hypocholesterolemic activity of vitamin A. Petersen (13) prevented the onset of hypercholesterolemia in cholesterol-fed chickens using dietary β-sitosterol, and the mechanism of this action has been investigated. It is now postulated that the inhibition occurs due to interference with the esterification of cholesterol which occurs during its transfer from the intestinal lumen to the lymph, probably at the site of transfer to the central lacteal (14). The possibility that vitamin A acts at the same point cannot be excluded but if the mode of action is similar then vitamin A is much the more potent in its action. Petersen *et al.* (15) showed that a ratio of 2:1 between β-sitosterol and cholesterol prevented completely the hypercholesterolemia due to the cholesterol. They found that the action of β-sitosterol decreased rapidly with lowering in concentration and that a ratio to cholesterol of 1:4 was only 50% effective. The same degree of protection was afforded by a vitamin A to cholesterol ratio of 1:1500. The fact that free fatty acids in the diet increased the hypercholesterolemia and that this effect was counteracted by the lingcod liver oil unsaponifiable material suggests that the esterification of the cholesterol may possibly be the site of action of the hypocholesterolemic material.

Acknowledgments

The author wishes to thank Mr. J. Topliff for his technical assistance, also Western Chemical Industries Ltd., Vancouver, B.C., for carrying out the vitamin A assays on the lingcod liver oils which they supplied.

References

1. B. Bronte-Stewart, A. Antonis, L. Eales, and J. F. Brock. Lancet, **1**, 521 (1956).
2. J. T. Anderson, A. Keys, and F. Grande. J. Nutrition, **62**, 421 (1957).
3. E. H. Ahrens, Jr., W. Insull, Jr., J. Hirsch, W. Stoffel, B. L. Petersen, J. W. Farquhar, T. Miller, and H. J. Thomasson. Lancet, **1**, 115 (1959).
4. A. P. DeGroot and S. A. Reed. Nature, **183**, 1191 (1959).
5. J. D. Wood and J. Biely. Can. J. Biochem. and Physiol. **38**, 19 (1960).
6. J. D. Wood and J. Biely. Nature, **185**, 473 (1960).
7. W. M. Sperry and M. Webb. J. Biol. Chem. **187**, 97 (1950).
8. L. A. Swain. J. Fisheries Research Board Can. **7**, 389 (1948).
9. P. R. Moore and C. A. Baumann. J. Biol. Chem. **195**, 615 (1952).
10. M. L. Karnovsky and W. S. Rapson. J. Soc. Chem. Ind. **22**, 138 (1946).
11. R. E. Reeves. J. Am. Chem. Soc. **63**, 1476 (1941).
12. G. Weitzel, H. Schön, F. Gey, and E. Buddecke. Hoppe-Seyler's Z. physiol. Chem. **304**, 247 (1956).
13. D. W. Peterson. Proc. Soc. Exptl. Biol. Med. **78**, 143 (1951).
14. L. Swell, E. C. Trout, Jr., R. Hopper, H. Field, Jr., and C. R. Treadwell. Ann. N.Y. Acad. Sci. **72**, 813 (1959).
15. D. W. Peterson, E. A. Shneour, N. F. Peek, and H. W. Gaffey. J. Nutrition, **50**, 191 (1953).

THE PHARMACOLOGY OF THE RAUWOLFIA ALKALOIDS CHANDRINE AND SERPAKRINE[1]

A. K. Reynolds, H. J. Presutti, and D. P. MacLeod

Abstract

The pharmacological properties of two new alkaloids of *Rauwolfia serpentina* have been studied with special reference to their cardiovascular actions. In several instances, comparative studies have been carried out with the clinically used alseroxylon fraction of *R. serpentina*. Chandrine and serpakrine do not profoundly influence the activity or behavior of intact animals unless administered in very large doses. They are completely devoid of the tranquilizing action that characterizes such bases as reserpine and rescinnamine. They do, however, exhibit marked hypotensive activity. The mechanism of this depressor action has not been clearly established. It is not seen in spinal animals, and there is no evidence of ganglionic blocking or peripheral adrenolytic action. The effects of chandrine or serpakrine per se on smooth muscle structures are not pronounced, but antiacetylcholine activity has been observed on these and other preparations. Their contribution to the actions of the clinically employed whole-root extracts is probably not very marked.

More than 30 alkaloids have been isolated from the several varieties of *Rauwolfia serpentina*. The methods of extraction and their characteristics have been very capably reviewed in numerous reports, for example those of Phillips and Chadha (10) and Holt and Costello (8). Likewise, several reviews of the pharmacology of these alkaloids have appeared, among them the excellent treatise of Bein (2). Crude preparations of *Rauwolfia* have been employed in the native medicine of India for centuries, but only in recent years have its principal spheres of therapeutic usefulness been established. The chief indications for the drug are in the management of hypertension and neuropsychiatric disorders. In view of the fact that alkaloidal concentrates and whole-root extracts are being employed clinically, it is important that the pharmacological properties of the constituent alkaloids be determined. In this connection, there appears to be some difference, as yet obscure, between the effects of the single alkaloid reserpine and the cruder *Rauwolfia* preparations. Indeed, hypertensive patients have expressed a preference for the latter (15). It is interesting to note that Rindani has reported protection against stress-induced adrenal hypertrophy by reserpine-free alkaloidal concentrates in contrast to the ineffectiveness of reserpine (13). The two alkaloids chandrine and serpakrine have been isolated from the Bengal variety of *Rauwolfia serpentina* by Rakshit (11). They are dark, yellowish-brown crystalline compounds which are slowly soluble in water. Their chemical configuration is currently being investigated. The chemical properties of chandrine have been reported (11).

Methods

Intact Animal Studies

Freshly prepared 1% solutions of the hydrochloride salts were employed throughout. These were administered to mice by intraperitoneal injection and

[1]Manuscript received February 1, 1960.
Contribution from the Department of Pharmacology, Dalhousie University, Halifax, N.S. This project was generously supported by a grant from the National Research Council of Canada.

to rabbits by the intravenous route. Observations were made on activity, behavior, respiration, and pupil size. Guinea pigs were exposed to aerosols of both alkaloids and observed for any signs of irritation or respiratory embarrassment.

Cardiovascular Studies

Cats ranging in weight from 2 to 3.5 kg and unselected as to sex were anesthetized with either chloralose or a chloralose–urethane mixture. Mean arterial pressure was recorded from the common carotid artery by means of a mercury manometer. Decerebrate and spinal preparations were also studied. Renal volume was recorded using a kidney oncometer-piston recorder system. For evoking pressor responses, adrenaline (4 μg per kg) and nicotine (15 μg per kg) were injected intravenously. The carotid sinus reflex was elicited by occluding the carotid artery caudad to the sinus for 45 seconds. Frog hearts were perfused *in situ* by means of a Greene cannula inserted in the inferior vena cava. Varying concentrations of the alkaloids were continuously perfused through the hearts using the single-bottle perfusion unit described by one of us (A.K.R.12). The effect of the alkaloids on isolated, electrically driven mammalian atria was studied according to the method of Dawes (6).

Studies on Smooth Muscle

The action of the alkaloids on isolated segments of guinea pig ileum were studied in the usual manner, and their effects on the cat intestine *in situ* were investigated using a 10-cm loop of jejunum filled with corn oil. Activity was recorded with a Marey tambour and drugs were injected into a cannulated femoral vein. The urinary bladder was cannulated via the urethra in female animals, and in male cats the cannula was tied into the urachus; the ureters were ligated. Activity was recorded by means of a tambour. The rabbit uterus was studied both in vitro and in vivo.

Studies on Antiacetylcholine Activity

Antiacetylcholine activity was studied in the usual manner on the guinea pig ileum and frog rectus abdominis preparations. In addition, the effect of these alkaloids was studied on both the *depressant* action of acetylcholine on spontaneously contracting isolated rabbit atria as described by Briscoe and Burn (5), and on the *stimulant* action of acetylcholine on atropine pretreated atria according to the method of Kottegoda (9). Antiacetylcholine activity was studied also in the rat using the suppression of "bloody tears" induced by carbaminoylcholine as a criterion.

Results

General Effects

Neither chandrine nor serpakrine exert any profound influence on activity or behavior in mice or rabbits in doses less than 75 mg per kg. In mice, doses in excess of 100 mg per kg produce general increased irritability and convulsions from which the animals recover provided that respiratory arrest does not occur during seizures. In surviving animals, recovery appears to be complete in 5 to 6 hours. In rabbits, doses of this magnitude cause serious respiratory

embarrassment, but complete recovery takes place provided adequate ventilation is maintained artificially. A transient miosis is always noted. This effect is characteristic of the alkaloid reserpine, but in the case of that alkaloid the action is a protracted one. Miosis is not seen with local application, and after systemic administration, it is abolished by atropine. Application of 1% solutions to the cornea does not produce any local anesthesia. Guinea pigs exposed to a 1% aerosol of either chandrine or serpakrine show no signs of local irritation or respiratory embarrassment.

Cardiovascular Studies

Both alkaloids are hypotensive in anesthetized cats and dogs, and lower the pressure in adrenaline or ephedrine-induced hypertension. Following intravenous injection, there is a sharp fall in mean arterial pressure, usually occurring about 30 seconds after injection. This 30-second latent period is known to be very characteristic of histamine liberators. However, the hypotensive action of chandrine and serpakrine is not influenced by the previous administration of adequate doses of the potent antihistaminic compound mepyramine (neoantergan) as shown in Fig. 1. Likewise, the action is not prevented by bilateral mid-cervical vagotomy or by atropinization. In some animals, following the initial drop in pressure, there is a partial recovery followed by a further fall and gradual return to a level somewhat below the original. This secondary fall is also seen in Fig. 1. The duration of the hypotensive action varied from 2 to 15 minutes. Respiration is not markedly affected by either of these alkaloids. The nature of the hypotensive action was influenced by the anesthetic employed. As shown in Fig. 2, the drop in pressure was preceded by a brief but very sharp pressor response when chloralose was used alone. This type of effect was seen in each of seven animals. In another series of seven animals anesthetized with a chloralose–urethane mixture, only the hypotensive effect was observed; no biphasic action was ever noted. These alkaloids caused a fall in blood pressure in each of three decerebrate preparations, but no hypotensive effect occurred in a series of five spinal animals. No ganglionic blocking or peripheral adrenolytic activity was observed in a series of six cats. The pressor response to injected nicotine or adrenaline was not altered by the

TABLE I

Blood pressure response to a dose of 5.0 mg per kg of chandrine in a series of eight cats

Animal No.	Preinjection pressure (mm Hg)	Fall in pressure*	% Fall
1	170	80	46
2	100	40	40
3	170	70	41
4	160	20	12
5	170	70	41
6	145	45	31
7	150	20	13
8	160	20	12

* Mean fall and S.D. 45 ± 25.
S.E.M. 8.8.

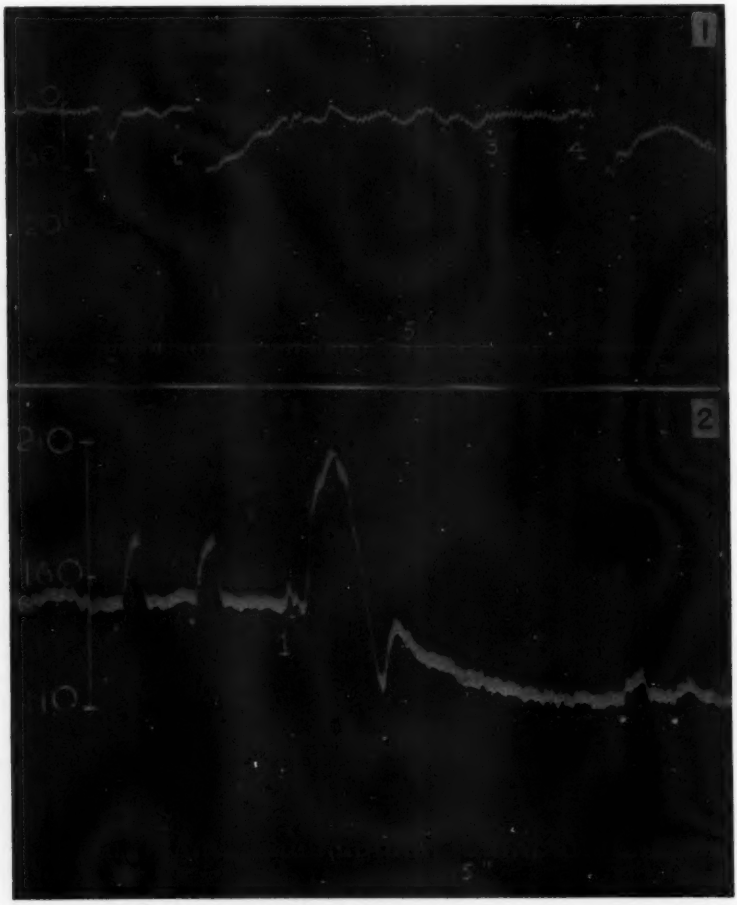


FIG. 1. The effect of chandrine, i.v. 5.0 mg per kg, on the blood pressure in the cat under chloralose–urethane. At 1, histamine; 2 mepyramine; 3 histamine; 4 chandrine. Time 5 seconds.

FIG. 2. The effect of chandrine on the carotid sinus pressor reflex in the cat under chloralose. At dots, carotid occlusion for 45 seconds. At 1, chandrine 5.0 mg per kg i.v. Time 15 seconds. Note the initial, sharp pressor response to chandrine (see text).

previous administration of depressor doses of chandrine or serpakrine. On the other hand, as can be seen in Fig. 2, the pressor response due to carotid occlusion was definitely suppressed, much more than would be anticipated as a result of the fall in arterial pressure alone. It should be pointed out that unlike reserpine, these alkaloids do not inhibit the carotid sinus reflex when administered in non-hypotensive doses. Table I shows the initial, acute blood pressure drop produced by a dose of 5.0 mg per kg of chandrine in one series of eight cats. It can be seen that there is considerable individual variation in response, a situation which has also been reported by Schlagel and Nelson in the response

of dogs to certain alkaloidal fractions of *Rauwolfia serpentina* (14). A decrease in renal volume parallels the fall in mean arterial pressure as shown in Fig. 3.

FIG. 3. The effect of serpakrine (S) and chandrine (C) on the blood pressure (upper) and renal volume (lower) in the cat under chloralose–urethane. Time 15 seconds.

On the perfused frog heart, the two alkaloids differ slightly in their actions. Serpakrine ($1:5\times10^4$) exerts a mild negative inotropic action with no concomitant chronotropic action. In the case of chandrine, a brief positive inotropic effect always precedes the depressant action on contractility. Continuous perfusion with the above concentration gradually leads to complete cardiac arrest in diastole. The effects are not altered by atropine. In electrically driven isolated rabbit atria exposed to concentrations of chandrine or serpakrine ranging from $1:2\times10^5$ to $1:5\times10^4$, definite prolongation of the refractory period was indicated. This can be seen in Table II where it will be noted that serpakrine and quinidine were almost equipotent with respect to both degree and duration of action. Of the two *Rauwolfia* alkaloids, the effect of serpakrine was somewhat stronger on this preparation. In Table II, column 5 gives the maximum depression (expressed as percentage of the control) of the rate at which the preparation could follow the stimulus. Column 6 gives the total duration of the depression. The alseroxylon fraction of *Rauwolfia serpentina* produced qualitatively similar effects on this preparation.

Antiacetylcholine Action

Serpakrine administered intraperitoneally in doses of 5.0 mg per kg 10 minutes prior to an injection of carbaminoylcholine prevented the appear-

TABLE II

The effect of chandrine, serpakrine, and quinidine on the isolated, driven rabbit atria

Compound	Concn.	Number of experiments	Average control MSR*	Average maximum depression (%)	Average duration depression (min)
Chandrine	$1:5\times10^4$	4	295 ± 40	25 ± 2.7	173 ± 15
	$1:10^5$	6	281 ± 40	19 ± 4.6	140 ± 49
	$1:2\times10^5$	3	256 ± 18	14 ± 1.0	68 ± 37
Serpakrine	$1:5\times10^4$	5	347 ± 34	37 ± 5.3	205 ± 50
	$1:10^5$	6	354 ± 12	21 ± 3.9	132 ± 40
Quinidine	$1:5\times10^4$	3	343 ± 46	38 ± 4.5	200 ± 10
	$1:10^5$	3	306 ± 99	28 ± 5.0	120 ± 15

* MSR = Maximum stimulation rate

ance of "bloody tears" invariably produced in control animals by 5.0 mg per kg of this choline ester. This antichromodacryorrhea action was observed in all of a series of 10 rats. Chandrine did not possess this action. On 10 segments of guinea pig ileum, serpakrine had little or no effect on normal activity, but it markedly suppressed the stimulant action of acetylcholine. The suppressant action of serpakrine was not a specific one for acetylcholine, however, since the stimulant action of the musculotropic spasmogen, barium chloride, was likewise inhibited. In sharp contrast to the action of serpakrine, chandrine always mildly augmented the action of acetylcholine on this preparation. This appeared to be an additive effect, since mild stimulation was produced by chandrine itself. The action was atropine-resistant. Gillis and Lewis (7) have reported an antiacetylcholine action on the guinea-pig ileum for the alkaloid reserpine, and Banerjee and Lewis (1) have made a similar observation on the isolated rabbit duodenum with regard to the alseroxylon fraction. Serpakrine and chandrine exhibited antiacetylcholine activity on another test object, the frog rectus abdominis muscle. Each alkaloid was tested on five preparations. Standard responses to acetylcholine (10^{-6}) in contact with the tissue for 90 seconds were obtained. The preparation was then exposed to either alkaloid before the next challenge with acetylcholine. It was then washed repeatedly and acetylcholine added to the bath at regular intervals until the contractions returned to normal or reached a stable plateau. In all experiments, both alkaloids markedly inhibited the stimulant action of acetylcholine. Thus, chandrine blocks the nicotinic effect of acetylcholine but not its muscarinic effect. In contrast, serpakrine blocks both these actions of acetylcholine. The action on this preparation was difficult to wash out, and as can be seen in Fig. 4, even after repeated washings, it required twice the concentration of acetylcholine to elicit a response comparable to that of the controls. Contractions induced by potassium chloride were not inhibited (Fig. 5). Reserpine and the alseroxylon fraction also exhibit antiacetylcholine activity on the rectus abdominis muscle (1, 7). Studies on isolated rabbit atria showed that serpakrine, chandrine, and the alseroxylon fraction depressed both the inhibitory action of acetylcholine on normal preparations and its stimulant action on atropine-pretreated atria.

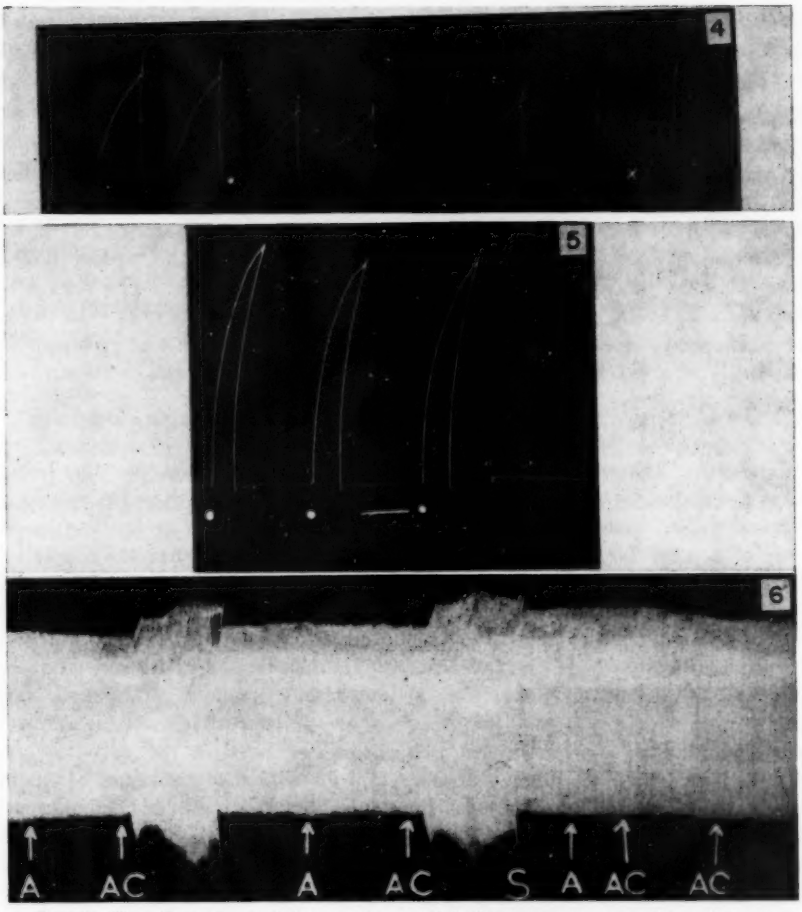


FIG. 4. The effect of serpakrine on the acetylcholine-induced contractions of the frog rectus abdominis muscle. Each contraction is due to acetylcholine. At dot, exposure for 3 minutes to serpakrine $1:10^5$. After repeated washing, double the original concentration of acetylcholine (at ×) was required to produce a contraction comparable to the control contractions induced by acetylcholine $1:10^6$.

FIG. 5. The lack of effect of serpakrine on potassium-induced contractions of the rectus abdominis muscle. All contractions were produced by KCl. Between second and third contractions, preparation was exposed to serpakrine for 3 minutes.

FIG. 6. The effect of serpakrine on the stimulant action of acetylcholine on atropinized, isolated rabbit atria. At A, atropine (12.5×10^{-6} g per ml): at AC, acetylcholine (5×10^{-5} g per ml): at S, serpakrine ($1:5\times10^4$).

Against the inhibitory action, serpakrine was the most potent. Chandrine was the weakest of the *Rauwolfia* preparations tested and was equipotent with quinidine. Also, serpakrine and the alseroxylon fraction were much more effective in restarting atria brought to complete standstill by acetylcholine than was quinidine. On the other hand, all three *Rauwolfia* preparations and

quinidine were about equally effective in suppressing the *stimulant* action of acetylcholine on atropine pretreated atria. Figure 6 shows a typical experiment with serpakrine.

Studies on the Gastrointestinal Tract, Urinary Bladder, and Uterus

In five cats, serpakrine and chandrine always exerted a brief stimulant action on the jejunum in vivo. In addition, chandrine initiated rhythmic movements in three other animals which showed no spontaneous activity. The action of these alkaloids on the intestine was not influenced by vagotomy but it was prevented by atropinizing the animal. In this respect, the action differed from that on the isolated terminal ileum of the guinea pig. The reason for this has not been determined. The urinary bladder showed a very brief period of diminished activity with little effect on tonus. The uterus in vivo was not markedly affected, but chandrine mildly stimulated the uterus in vitro.

Discussion

The two *Rauwolfia* alkaloids chandrine and serpakrine do not belong pharmacologically with the so-called tranquilizing bases such as reserpine and rescinnamine. Indeed, their effects on activity and behavior are manifested only after very large doses — doses which are excitant rather than sedative. Very few of the more than 30 alkaloids isolated thus far from *Rauwolfia serpentina* possess the unique type of sedative action characteristic of reserpine and perhaps also of such compounds as meprobamate, chlorpromazine, and others. Hypotension is perhaps the most characteristic effect of chandrine and serpakrine. The exact mechanism of this action has not been established. The two alkaloids exhibit no ganglionic blocking or peripheral adrenolytic activity. Very mild parasympathomimetic activity is suggested in the pupillary and gastrointestinal effects, but this does not appear to extend to the cardiovascular system. For example, unlike reserpine, no bradycardia has ever been observed either in the intact animal or in isolated perfused hearts. Histamine liberation is not a factor in the depressor action, since it occurs even after administration of potent antihistaminic compounds. The hypothalamus has been generally regarded as the principal site of action of reserpine, the depressor effect being ascribed to a loss of sympathetic dominance through an action on this integrating center. However, the studies of Bhargava and Borison, in which they employed the stereotaxic technique to advantage, strongly implicate the medulla as the most important site of action. Furthermore, these investigators observed a distinct difference in the effects of reserpine on the one hand and the alseroxylon fraction on the other. In contrast to reserpine, the latter exhibited a definite spinal component of action as well as action at the medullary and hypothalamic levels (3, 4). However, in view of the adrenaline-depleting action of reserpine, a careful reconsideration of the mechanism of the hypotensive effect of *Rauwolfia* alkaloids would appear to be essential.

The antiacetylcholine activity observed in various preparations is interesting in that it has also been reported for both reserpine and the alseroxylon fraction by other investigators. Only on the rectus muscle did chandrine exhibit this

property. Chandrine differs from serpakrine in other respects as well. It tends to exert a mild stimulant action on the smooth muscle of the intestine and uterus in vitro, an action not shared by serpakrine. Moreover, on the perfused frog heart, chandrine invariably shows an initial stimulant action prior to its negative inotropic action.

Chandrine and serpakrine are present in the whole-root extracts that are employed clinically. This type of *Rauwolfia* preparation finds more extensive use in the treatment of hypertension than in the management of neuropsychiatric disorders. In view of the relatively large doses of chandrine and serpakrine required to elicit a marked depressor effect, it would seem that the contribution of these two alkaloids to the antihypertensive action of the whole-root preparations is slight.

Acknowledgments

The authors wish to express their sincere thanks to Dr. Walter C. Murphy of Ciba Company Ltd., Montreal, for a generous supply of reserpine, and to Drs. J. W. Stutzman and G. E. Cronheim of Riker Laboratories, Los Angeles, California, for their interest and a generous supply of rauwiloid — the alseroxylon fraction used in this study.

References

1. J. N. Banerjee and J. J. Lewis. J. Pharm. and Pharmacol. **7**, 50 (1955).
2. H. J. Bein. Pharmacol. Revs. **8**, 435 (1956).
3. K. P. Bhargava and H. L. Borison. J. Pharmacol. **115**, 464 (1955).
4. K. P. Bhargava and H. L. Borison. J. Pharmacol. **119**, 395 (1957).
5. S. Briscoe and J. H. Burn. Brit. J. Pharmacol. **9**, 421 (1954).
6. G. S. Dawes. Brit. J. Pharmacol. **1**, 90 (1946).
7. C. N. Gillis and J. J. Lewis. J. Pharm. and Pharmacol. **8**, 606 (1956).
8. W. L. Holt and C. H. Costello. J. Am. Pharm. Assoc. Sci. Ed. **43**, 144 (1954).
9. S. R. Kottegoda. Brit. J. Pharmacol. **8**, 83 (1953).
10. D. D. Phillips and M. S. Chadha. J. Am. Pharm. Assoc. Sci. Ed. **44**, 553 (1955).
11. B. Rakshit. Indian Pharmacist, **10**, 84 (1954).
12. A. K. Reynolds. J. Am. Pharm. Assoc. Sci. Ed. **41**, 398 (1952).
13. T. H. Rindani. Arch intern. pharmacodynamie, **102**, 465 (1955).
14. C. A. Schlagel and J. W. Nelson. J. Am. Pharm. Assoc. Sci. Ed. **43**, 505 (1954).
15. R. W. Wilkins. Ann. N.Y. Acad. Sci. **59**, 1 (1954).

EXPERIMENTAL EXAMINATION OF THE MECHANISM OF PYRAMIDON EFFECT

III. EFFECT OF HIGH PYRAMIDON DOSES ON THE GLUTAMIC ACID – OXALOACETIC ACID TRANSAMINASE ACTIVITY IN SERUM[1]

Á. Gy. Fazekas, I. Gy. Fazekas, and B. Rengei

Abstract

In five rabbits, the changes of glutamic acid – oxaloacetic acid transaminase (SGOT) activity in serum were examined after a Pyramidon treatment in which 75% of the single lethal dose was given for 5 days, divided in three doses. Depending on the individual sensitivity, SGOT activity became from 3 to 8 times higher. This increase appeared to be a result of extensive renal lesions.

In earlier examinations, high doses of Pyramidon resulted in severe impairment of renal function, and fatty degeneration of the liver. It has been shown that myocardial and hepatic lesions of different origin, e.g. myocardial infarction (1), hepatitis (2), acute and chronic alcohol intoxication (3), carbon tetrachloride poisoning (4), and nicotine poisoning (5) are attended by increased SGOT activity. Our quoted observations and the above-mentioned literature data induced us to examine the effect of Pyramidon on SGOT activity.

Method

Female chinchilla rabbits bred in this Institute were used. The rabbits weighed from 2300 to 2920 g. Their food was 60 g of oats, 60 g of dried clover, per day, and water ad libitum. The single lethal dose of Pyramidon for rabbits is 0.4 g/kg subcutaneously (6). The rabbits were given one-fourth of this dose, i.e. 0.1 g/kg, three times a day, at 9, 14, and 19 o'clock. Thus, they received daily 75% of the single lethal dose, divided in three parts. The drug was administered for 5 days in a 5% aqueous solution, subcutaneously.

Blood was withdrawn from a marginal ear vein, in the morning, before the first Pyramidon injection. After the termination of treatment, blood was taken daily at the same time.

SGOT activity was determined by Dubach's method (7). In this procedure, the amino group of the aspartic acid added to the enzyme-containing substance is transferred, by the enzyme, to the α-ketoglutaric acid, while oxaloacetic acid and glutamic acid are formed. Then, oxaloacetic acid is, by aniline citrate, converted to pyruvic acid that forms a hydrazone with dinitrophenylhydrazine. The hydrazone is shaken with toluol. Thereafter it forms with alcoholic potassium hydroxide a red complex, the hue intensity of which is proportional to the amount of pyruvic acid, and, thus, to the activity of transaminase.

The unit of transaminase is defined as the activity of 1 ml of a serum giving rise to the formation of 1 μg of pyruvic acid in 20 minutes at a temperature of 25 ± 2 C°.

[1]Manuscript received February 3, 1960.
Contribution from the Institute of Forensic Medicine, Medical University of Szeged, Hungary.

Results and Discussion

The results of the transaminase determinations are reported in Table I. The data show that Pyramidon increased the activity of the enzyme in all five animals. The increase of SGOT activity began on the first day of treatment, and arrived, after a gradual progress, at its peak on the last day. The increase was not equal in all animals. Rabbit No. V displayed an increase of 151 units (520%), rabbit No. IV only 49 units (357%). The inference is therefore warranted that rabbits display to Pyramidon an individually different sensitivity.

TABLE I

Changes in the SGOT activity of rabbits under the effect of Pyramidon (3×0.1 g/kg subcutaneously, for 5 days)

No.	Transaminase activity						SGOT activity after treatment		Max. increase of SGOT activity	Died after last treatment
		During treatment								
	Before treatment	1st day	2nd day	3rd day	4th day	5th day	2nd day	5th day		
I	29	88	139	149	149	159	98	—	130	At 5 days
II	19	78	98	108	139	139	108	78	120	At 6 days
III	10	29	39	88	78	88	39	29	78	Survived
IV	19	58	48	48	58	68	29	29	49	Survived
V	29	68	128	169	180	180	—	—	151	At 1 day

Two days after the last treatment considerably lower SGOT values were obtained. Rabbit No. IV yielded then a nearly normal activity. On the 5th day, activity was still more reduced. Rabbit No. V died on the next, No. I on the 5th, No. II on the 6th day after the termination of treatment. In all, death was due to uraemia, as we reported earlier (8). For instance, in rabbit No. V the N.P.N. level was 155 mg% before death.

The increase of SGOT activity was undoubtedly due to the Pyramidon-induced renal and hepatic lesions, because necrotizing nephrosis with severe glomerular damage (9) and fatty degeneration of the liver parenchyma (10) were observed. Strikingly, rabbits Nos. I and II died, unlike rabbit No. V, after the termination of treatment with a considerably lower activity of transaminase. As mentioned, the animals died of uraemia. The question arises, in which way did the animals die in the period of decreasing SGOT activity, i.e. in the period of "convalescence".

First, the fact should be emphasized that no new damage arose after the last dose of Pyramidon. In the injured areas regeneration began, mainly in the liver, whilst the irreversible renal lesion resulted in the progressive increase of N.P.N. substances, until lethal uraemia developed, simultaneously with the decrease of SGOT activity.

In our view, it is chiefly the extension and severity of renal damage that caused the Pyramidon-induced elevation of SGOT activity. This view is supported by the fact that those rabbits survived (Nos. III and IV) in which the least increase of SGOT activity occurred. Their renal lesions could not be

severe, since they did not develop uraemia. A further evidence for our view was the order in which the rabbits died: Nos. V, I, II, i.e. greater increase of SGOT activity designating a more severe renal lesion was associated with earlier uraemia and earlier death.

Obviously, the repeated administration of high doses of Pyramidon led to the increase of SGOT activity. The increase was, however, different, depending on the individual sensitivity and the resistance to Pyramidon of the organism. Our earlier observations stating that the repeated administration of high Pyramidon doses results in renal and hepatic damage have been confirmed by these experiments.

References

1. J. S. La Due, F. Wróblewski, and A. Karmen. Science, **120**, 497 (1954).
2. F. Wróblewski and J. S. La Due. Ann. Internal Med. **43**, 345 (1955).
3. N. U. Bang, K. Iversen, T. Jagt, and S. Madsen. J. Am. Med. Assoc. **168**, 157 (1958).
4. D. W. Molander, F. Wróblewski, and J. S. La Due. J. Lab. Clin. Med. **43**, 345 (1955).
5. L. Veress and B. Rengei. Deut. Z. ges. gerichtl. Med. **49**, 169 (1959).
6. R. Kobert. Z. klin. Med. **62**, 57 (1907).
7. A. C. Dubach. Schweiz. med. Wochschr. **87**, 185 (1957).
8. I. Gy. Fazekas, Á. Gy. Fazekas, and F. É. Bertok. In preparation.
9. I. Gy. Fazekas, Á. Gy. Fazekas, and F. É. Bertok. In preparation.
10. I. Gy. Fazekas, Á. Gy. Fazekas, and F. É. Bertok. In preparation.

STUDIES ON WHEAT PLANTS USING CARBON-14 COMPOUNDS

XIII. INCORPORATION OF α-AMINO ADIPIC ACID-6-C^{14} IN WHEAT PLANTS[1]

R. NATH[2] AND W. B. MCCONNELL

Abstract

α-Amino adipic acid-6-C^{14} was injected into the stems of wheat plants and the distribution of carbon-14 in the mature plants was studied. About 80% of carbon-14 injected was recovered in the plants, the kernels alone accounting for 56%.

Glutamic acid, labelled predominantly in carbon-5, was the most radioactive compound isolated from wheat kernels, a result in accord with the concept that acetate-1-C^{14} was a product of α-amino adipic acid-6-C^{14} metabolism in the wheat plant.

The specific activity of the lysine of the protein was low and hence the data provide no evidence that α-amino adipic acid is a precursor of lysine in wheat.

Introduction

Certain aspects of amino acid biosynthesis in wheat plants have been studied in this laboratory by examining the carbon-14 distribution in mature kernels labelled with suitable tracers at late stages of growth (1). Acetate-1-C^{14} incorporation, in particular into amino acids such as glutamic acid, has been investigated in some detail and a characteristic pattern for the resulting carbon-14 distribution has been observed (2, 3). This approach has furnished a means for detecting acetate formation during the metabolism of other carbon-14 labelled substances and has been utilized to show that pyruvate is extensively decarboxylated in wheat plants (4, 5).

Another compound that may yield acetate is α-amino adipic acid. This possibility is suggested by the work of Miller and Bale (6) with animals which indicates that α-amino adipic acid lies on a pathway leading from lysine to glutamic acid and that acetate is a probable intermediate. It appeared feasible to study the incorporation of labelled α-amino adipic acid by techniques now being used, the results being more amenable to interpretation because of earlier investigations with acetate-1-C^{14}. The fate of α-amino adipic acid when administered to wheat is of particular interest also because the compound has been suggested as a precursor of lysine (7). The low lysine content of wheat protein is a matter of concern (8, 9), and, since wheat is an important element in human diet, means for overcoming the nutritional deficiency are needed. The mode of lysine biosynthesis in wheat is therefore a question of general interest and, except for recent work by Vogel (10), it is one which has hitherto received little attention.

This communication reports observations on the fate of radioactive carbon injected into maturing wheat plants in the form of α-amino adipic acid-6-C^{14}.

[1]Manuscript received April 29, 1960.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan.
Issued as N.R.C. No. 5745.

[2]National Research Council of Canada Postdoctorate Fellow 1957–59. Present address: Department of Biochemistry, School of Medicine, University of California, San Francisco, California.

Experimental Methods

Thatcher wheat plants were seeded outdoors on May 18, and 81 days later (August 7) α-amino adipic acid-6-C^{14}* in aqueous solution was administered to the plants (2). The material was injected into the hollow of the top internode on one tiller of each of 10 plants. The dose per tiller was 0.2 ml of solution containing 2.46 mg of α-amino adipic acid-6-C^{14} (1.37 μc of carbon-14).

Labelled tillers were harvested on August 19 (93 days after seeding, 12 days after injecting) at which time they had fully matured. The plants were air-dried, separated into appropriate plant parts, and after a further drying period of 1 day in vacuum at 40° C they were ground to pass a 40-mesh screen. The ground kernels were further separated into arbitrary fractions designated as starch, gluten, salt-soluble protein, ether-soluble material, and bran (or residue) according to a previously adopted scheme (11).

Certain amino acids were isolated (2, 12) from acid hydrolyzates of gluten and each of these was decarboxylated with ninhydrin for separate recovery of carbon-1 (2, 13). The specific activities of the amino acids and the products obtained in their degradation (5) were determined by combusting the material to carbon dioxide and counting the gas with the Dynacon vibrating reed electrometer (Nuclear Chicago Corp.), using the 250-ml counting chamber. The activity of other materials was obtained by plate counting at infinite thickness using an end-window counter (Micromil, Nuclear Chicago Corp.). The factor obtained for the conversion of the data of plate counting rate to C^{14} content, in the earlier work on pyruvates (5), was used in the present study. Carbon-5 of glutamic acid was obtained by the Schmidt reaction (14).

Experimental Results and Discussion

The carbon-14 content of various plant parts harvested (Table I) was measured by plate counting and errors as large as 10% may be involved. Also, quantitative differences in the labelling of individual plants occur, especially in parts near the site of injection, and the number of tillers used was not large enough for satisfactory statistical treatment.

TABLE I

Carbon-14 content of the plant parts

Plant part	Weight, g/plant	Specific activity, c.p.m.	C^{14}, μc/plant	% of C^{14} injected
Stem	.31	10,300	.242	17.7
Leaf and sheath	.17	1,800	.023	1.7
Kernel	1.23	7,600	.760	55.7
Chaff	.27	2,600	.054	3.9
Rachis	.07	2,500	.013	1.0
	2.05		1.092	80.0

About 80% of the carbon-14 injected could be accounted for in the upper portion of the labelled plants. This is unusually high in comparison with similar

*α-Amino adipic acid-6-C^{14} used in the work was kindly supplied to us by Dr. L. L. Miller, University of Rochester, Rochester, New York.

studies using other tracers (2, 5, 11). Kernels contained more than one half (56%) of injected tracer but an appreciable quantity (17%) remained in the internode of the stem injected. No experiments were done to determine the form of carbon-14 in these stems and it is possible that some of it remained as unused α-amino adipic acid, perhaps adsorbed on interior tissue surfaces of the straw. Other plant parts collected contained smaller amounts of radioactivity. Plant portions below the top node were not examined.

Data obtained after fractionation of the kernels are given in Table II. All kernel components isolated contained appreciable amounts of carbon-14 but protein was clearly the most radioactive. Thus, although gluten isolated constituted only 11.4% of kernel weight it contained more than 30% of the radioactivity. The salt-soluble protein was appreciably radioactive but since it is present in small amounts it only contained 5% of carbon-14. The starch, representing 59% of the kernel weight, contained only 44% of tracer in kernels. Other fractions were low in carbon-14 and it appears that about 10% of the radioactivity was discarded during fractionation as soluble material.

TABLE II

Recovery of carbon-14 from kernel fractions

Fraction	Weight recovered, g/g kernels	Specific activity, mμc/mmole CO_2	C^{14} in fraction, μc
Kernels	1.000	19*	.62
Starch	.589	12	.27
Gluten	.114	36.5	.19
Salt-soluble protein	.040	21	.03
Ether-soluble material	.013	9	.01
Bran	.095	12	.05
	.851		.55

*Estimated by plate counting.

The specific activities of amino acids isolated and of their carboxyl groups are given in Table III. The specific activity of lysine is considerably lower than that of many other amino acids isolated from the protein and is, in fact, lower than that of kernel carbon as a whole. However, if, as reasoned in designing the experiment, α-amino adipic acid is a natural precursor of lysine in wheat the specific activity of the lysine in the gluten could well have been considerably greater than that of other kernel components. The results therefore do not support the idea of lysine biosynthesis in wheat by a pathway involving α-amino adipic acid as a precursor.

It is significant that a particularly large amount of radioactivity was found in glutamic acid and in the related amino acids, proline and arginine. Carbon-1 of these amino acids is also appreciably radioactive. It was estimated (glutamic acid content of gluten taken as 35.5% (15)) that glutamic acid in 1 g of wheat would contain 0.10 μc of carbon-14. This would be more than one half of the carbon-14 in the gluten, about one sixth of carbon-14 in the kernels and one eleventh of the total carbon-14 injected. It was found that carbon-5 of the glutamic acid had a specific activity of 250 mμc/mmole of carbon dioxide.

TABLE III

Labelling amino acids of wheat gluten

Amino acid	Specific activity, mμc/mmole CO_2	
	Total	1-C
Glutamic acid	74	85
Proline	55	86
Arginine	53	49
Aspartic acid	33	50*
Threonine	14	20
Glycine	17	20
Serine	17	18
Alanine	14	23
Leucine	7	—
Isoleucine	3	5
Valine	3	2
Methionine	1	—
Histidine	2	—
Lysine	5	—

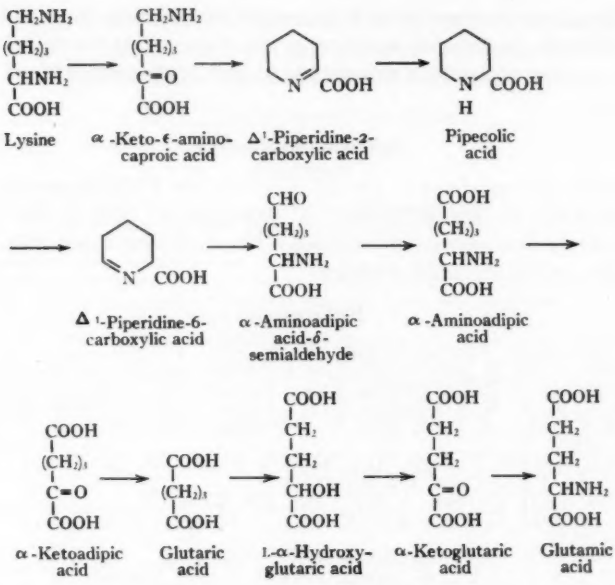
*Carbons 1 and 4 combined.

This carbon thus accounts for about 67% of the radioactivity in glutamic acid and since carbon-1 (specific activity, 85 mμc/mmole CO_2) contained 23% of the carbon-14 the terminal carbons accounted for 90% of the total carbon-14 in the glutamic acid. The carbon-14 distribution is similar to that obtained in parallel experiments with acetate-1-C^{14} (3, 16).

Aspartic acid was found to contain about 75% of its activity in carbons 1 and 4, a result also similar to that obtained with acetate-1-C^{14} feeding (3). Proline was found to be strongly labelled in carbon-1 whereas arginine had lower activity in the carboxyl groups. The latter two amino acids have consistently been found to parallel glutamic acid in labelling experiments with wheat plants (17).

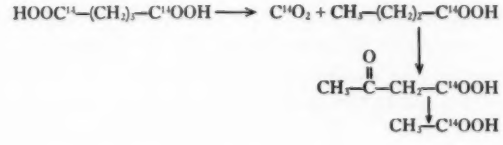
The results are very similar in many respects to those obtained by Miller and Bale (6) after feeding lysine-6-C^{14} to rats and dogs. They found, for example, that glutamic acid, aspartic acid, and arginine were labelled and that carbon-5 of glutamic contained 71% of the total radioactivity in this amino acid whereas the carbon-1 contained 25% of the carbon-14. They also noted localization of carbon-14 in the carboxyl groups of aspartic acid. These authors also pointed out the similarity of the results to those to be expected from acetate-1-C^{14} utilization by way of the tricarboxylic acid cycle and conclude that serious consideration must be given to the possibility that carbon-6 of lysine is incorporated into glutamic acid by way of a two-carbon fragment. Similarly, in the present experiments, it seems probable that a two-carbon fragment, behaving as acetate, plays a major role in the conversion of carbon-6 of α-amino adipic acid to glutamic acid.

Miller and co-workers extended their studies of lysine metabolism in animals (18) and as a result of these and other researches a more direct route from lysine to glutamic acid, which involves α-amino adipic acid as an intermediate, has been suggested. The scheme is shown below (19):



The production of glutamic acid from α-amino adipic acid has been confirmed in recent animal experiments by Rothstein and Greenberg (20).

The occurrence of a similar scheme in wheat plants could account for the parallel features in Miller and Bale's animal experiments with lysine-6-C^{14} and present data from α-amino adipic acid-6-C^{14}. Although it is probable that α-amino adipic acid is on a metabolic pathway leading from lysine to glutamic acid there is no evidence that the conversion of a five-carbon intermediate such as glutaric acid has occurred in the wheat plants in the manner shown in the diagram. Since glutaric acid is a symmetrical compound it should, if labelled in a carboxyl group, give rise to glutamic acid labelled equally in both carboxyl groups. Although direct utilization of some glutaric acid for glutamic acid formation is not altogether ruled out, the results are adequately explained by assuming acetate formation in a manner such as indicated by Rothstein and Miller (21) in the following equation:



It is concluded that incorporation of carbon-14 from α-amino adipic acid-6-C^{14} into the glutamic acid of wheat kernel proteins occurs largely by way of acetate-1-C^{14} and that direct formation of the carbon chain of glutamic acid from the intermediate glutaric acid is of minor importance, if indeed it occurs

at all. The conclusion would be in accord with recent findings of Hobbs and Koeppe (22), who concluded that in rats the major catabolic route of glutaric acid is via acetate rather than a direct conversion of the carbon chain to α-ketoglutaric acid.

Acknowledgments

The authors are indebted to Dr. D. Knott of the Field Husbandry Department, University of Saskatchewan, for wheat plants used in this work, and to Mr. J. Dyck for carbon-14 analyses. The technical assistance of Mr. A. Khan is also gratefully acknowledged.

References

1. W. B. McConnell. Chem. in Canada, **10**, 27 (1958).
2. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **35**, 357 (1957).
3. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **35**, 365 (1957).
4. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **36**, 381 (1958).
5. W. B. McConnell, R. Nath, and J. F. T. Spencer. Can. J. Biochem. and Physiol. **38**, 33 (1960).
6. L. L. Miller and W. F. Bale. Arch. Biochem. Biophys. **48**, 361 (1954).
7. E. Windsor. J. Biol. Chem. **192**, 607 (1951).
8. E. Geiger and H. J. Dunn. J. Biol. Chem. **178**, 877 (1949).
9. H. R. Rosenberg, E. L. Rohdenburg, and J. T. Baldini. Arch. Biochem. Biophys. **49**, 263 (1954).
10. H. J. Vogel. Proc. Natl. Acad. Sci. **45**, 1717 (1959).
11. W. B. McConnell and L. K. Ramachandran. Can. J. Biochem. and Physiol. **34**, 180 (1956).
12. C. H. W. Hirs, S. Moore, and W. H. Stein. J. Am. Chem. Soc. **76**, 6063 (1954).
13. D. D. Van Slyke, D. A. MacFayden, and P. Hamilton. J. Biol. Chem. **141**, 671 (1941).
14. E. H. Mosbach, E. F. Phares, and S. F. Carson. Arch. Biochem. Biophys. **33**, 179 (1951).
15. J. W. Pence, D. K. Mecham, A. H. Elder, J. C. Lewis, N. S. Snell, and H. S. Olcott. Cereal Chem. **27**, 335 (1950).
16. W. B. McConnell and E. Bilinski. Cereal Chem. **35**, 66 (1958).
17. W. B. McConnell and E. Bilinski. Can. J. Biochem. and Physiol. **37**, 933 (1959).
18. M. Rothstein and L. L. Miller. J. Am. Chem. Soc. **75**, 4371 (1953).
19. A. Meister. Biochemistry of the amino acids. Academic Press Inc., New York. 1957. p. 362.
20. M. Rothstein and D. M. Greenberg. J. Am. Chem. Soc. **81**, 4756 (1959).
21. M. Rothstein and L. L. Miller. J. Biol. Chem. **199**, 199 (1952).
22. D. C. Hobbs and R. E. Koeppe. J. Biol. Chem. **230**, 655 (1958).

EFFECTS OF INTRAVENOUS INJECTIONS OF THE ANTICOAGULANT FRACTION OF INCUBATED FIBRINOGEN ON BLOOD COAGULATION[1]

D. C. TRIANTAPHYLLOPOULOS

Abstract

AFIF (Anticoagulant Fraction of Incubated Fibrinogen precipitated between 25 and 50% ammonium sulphate saturation) prepared from Armour's incubated bovine fibrinogen was injected in a dosage of 46–106 mg (mean: 66.5 mg) tyrosine per kilogram body weight in the external jugular vein of 10 rabbits. Blood samples were withdrawn at ½- or 1-hour intervals until the aspirated blood began showing signs of coagulation. The anticoagulant effect was manifested immediately and lasted for 2½ to 5½ hours in the animal. Although the blood aspirated during this time remained unclotted even after 24 hours, the surgical wound in the neck of the animal did not bleed and autopsies revealed no sign of internal haemorrhage. The bleeding time, however, was found prolonged when tested by cutting the marginal vein of the ear. The level of the coagulation factors was determined both in oxalated specimens and in specimens with no anticoagulant added. Both kinds of sample showed: (1) clottable fibrinogen content normal, thus excluding fibrinolysis as the cause of the anticoagulant effect; (2) thrombin clotting time of infinity; (3) one-stage prothrombin time longer than 60 seconds; (4) no correction of the infinite thrombin clotting time of oxalated specimens following the addition of 0.25 mg protamine per ml. This makes unlikely any appreciable release of heparin by the animal. However, oxalated and non-oxalated specimens differed in the following respects: (1) prothrombin time determined after adsorption and mixing of the eluate with adsorbed plasma was found to be normal for the oxalated blood but variably increased for the non-oxalated specimen (10–34.5 seconds); (2) the plasma precursors of plasma thromboplastin were normal in oxalated but very low in native specimens. The serum precursors of plasma thromboplastin were normal.

In previous work (1, 2, 3) it was shown that the fraction of incubated fibrinogen precipitated between 25 and 50% ammonium sulphate saturation inhibits blood coagulation in vitro. The concentration required to produce this effect was found to be 46 ± 17 mg tyrosine equivalent of human AFIF per 100 ml human native plasma. The concentration of factor V and the plasma precursors of plasma thromboplastin were found to be lowered in blood rendered incoagulable by the addition of AFIF. On the other hand, the concentration of factor VII, serum precursors of plasma thromboplastin, and fibrinogen remained normal. Thrombin, although inhibited, was not destroyed by AFIF, even when incubated with the anticoagulant. The inhibition of the coagulation of fibrinogen by thrombin was found to be competitive in nature and had the following characteristics:

(*a*) it decreased after incubation at 46° C and was abolished at 55° C;

(*b*) it was proportional to the pH of the reaction mixture;

(*c*) it was reduced by calcium and magnesium in concentrations up to 10 m*M* in a manner proportional to the effect of these ions on the thrombin time of plain fibrinogen.

[1]Manuscript received March 15, 1960.

Contribution from the Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta. Supported by grants from the National Research Council and the Alberta Heart Foundation. Presented at the January, 1960, 14th Annual Meeting of the Western Regional Group of the National Research Council, Banff, Alberta.

It was also found that AFIF reduced the procoagulant effect of protamine on the thrombin time of fibrinogen and that it protected the latter from precipitation by protamine. The hydrolytic action, however, of thrombin on tosyl-arginine methylester was not affected by AFIF. In view of the origin of AFIF and its ability to inhibit blood coagulation in the test tube, it was decided to test its effectiveness as an anticoagulant in vivo.

Materials and Methods

Preparation of AFIF

One-gram lots of Armour's bovine fraction I were dissolved in 500 ml normal saline containing 25 ml 0.2 *M* imidazole buffer, pH 7.3, and 25 ml 0.03 *M* disodium-EDTA. The concentration of fibrinogen was purposely kept low in order to take advantage of the faster fibrinolysis that occurs at low concentrations (4). Disodium-EDTA was added to ensure complete removal of the calcium ions since the latter inhibit fibrinolysis (5). The mixture was passed through a Seitz filter and incubated under sterile conditions for 12 days. At the end of this period the fibrinogen did not clot upon dilution with distilled water and addition of thrombin (20 units per ml). Following ammonium sulphate fractionation, AFIF (fraction between 25 and 50% saturation) was Seitz filtered, dialyzed under sterile conditions for 24 hours, and lyophilized. The dried AFIF was dissolved in 10–20 ml normal saline and its tyrosine content determined. The osmolarity of such a solution was found by the freezing point depression method to be 71 milliosmoles per liter (3300 mg tyrosine as AFIF per liter). All AFIF solutions were stored at −25° C until used (2–3 days).

Incubated Human Fibrinogen

Two experiments were performed using incubated human fibrinogen instead of AFIF of bovine origin. Two grams of sterile human fraction I were dissolved into 200 ml of pyrogen-free distilled water and incubated at 37° C under sterile conditions. Samples tested during the first 2 weeks of incubation showed a considerable amount of clottable fibrinogen present. At the 18th day of incubation the formation of a flocculent precipitate was observed upon dilution with distilled water (1:4) and addition of thrombin. At this time 31 ml of the incubated fibrinogen mixed with 2.5 ml calcium gluconate – glucoheptonate (Abbott) was slowly injected into the external jugular vein of a 3.4 kg rabbit. Calcium was added in order to prevent the ventricular fibrillation which frequently follows administration of citrate through the jugular vein (6).

The rest of the fibrinogen solution was kept in the incubator for an additional 11 days and was tested again for coagulability. This time, addition of thrombin after dilution of the sample with distilled water did not clot the fibrinogen. Calcium chloride, however, added to a concentration of 10 m*M*, together with thrombin, produced a fine flocculent precipitate. Saturation of this fibrinogen solution with ammonium sulphate up to 25% yielded a heavy precipitate but further saturation of the supernatant to 50% produced only a small amount of precipitable material. This 29 days' incubated sterile fibrinogen was dialyzed

in the cold under sterile conditions for 2 consecutive days against two changes of distilled water, 2 liters each. It was finally lyophilized and redissolved in 15 ml pyrogen-free normal saline.

Experimental Animals

These were cross-bred domesticated rabbits. It was found in preliminary experiments that the minimal concentration of AFIF able to prevent the coagulation of the shed blood of these rabbits after intravenous injection was the same as the one required to inhibit coagulation in vitro, i.e. 86 (54–128) mg tyrosine equivalent of AFIF per 100 ml blood. From the results of the in vitro tests, and assuming that the blood volume of rabbits is 70 ml per kg of body weight (7), the amount of each AFIF preparation necessary to achieve complete incoagulability was calculated and injected into seven rabbits. Three other animals received a smaller amount of anticoagulant.

The rabbits were anaesthetized by injecting 27 mg of Nembutal per kg body weight through the marginal vein of the ear. Through a midline cervical incision, the right external jugular vein was exposed and catheterized using either polyethylene tubing or the softer "Infant Feeding Tubes".[2] In two unanaesthetized rabbits the AFIF preparation was injected directly into the marginal vein of the ear. Through the catheter, AFIF or incubated human fibrinogen was administered, blood specimens were aspirated and in some cases blood was transfused. After the conclusion of the experiment, the catheter was removed, the vein ligated, and the wound sutured.

The animals which survived were kept under observation for several days after the experiment and it was noticed that the healing of the surgical wound was rapid and uneventful. The faeces and urine of these rabbits remained clear of any admixture of blood throughout the period of observation. The thoracic and abdominal viscera of the animals which died were examined after death.

Thrombin Clotting Time (8) was measured by adding 0.1 ml of a thrombin solution[3] in distilled water (10 N.I.H. units per ml) to 0.2 ml plasma. The time required for the formation of a clot was determined.

One-stage Prothrombin Time and Fibrinogen Concentration were determined according to established methods (8). The prothrombin time was also determined by the adsorption and elution technique (8). This was done in order to avoid the presence of AFIF in the reaction mixture. Prothrombin, factor VII, and factor X (Stuart factor) (9) were adsorbed on tricalcium phosphate. For elution, 0.2 *M* sodium citrate, one fifth the original plasma volume, was used. One volume of the eluate was mixed with nine volumes adsorbed rabbit plasma and the one-stage prothrombin time of the mixture was determined.

The Thromboplastin Generation Test (8) was performed using the platelet substitute of Bell and Alton, prepared from rabbit brain thromboplastin and tricalcium-phosphate-adsorbed oxalated plasma instead of alumina-citrated plasma. Human oxalated plasma served as substrate.

[2]Manufactured by C. R. Bard, Inc., Summit, New Jersey, U.S.A.
[3]Thrombin, Topical, Parke, Davis & Co., Ltd.

Results

AFIF prepared from Armour's bovine fraction I was injected into 10 rabbits and incubated human fraction I into two others (Table I). Seven out of the 10 animals which were injected with bovine AFIF received an adequate amount

TABLE I

Effect of intravenous injections of AFIF or incubated fibrinogen on the coagulability of blood

Rabbit No.	Amount of anticoagulant injected		Blood transfused, ml	Effect of anticoagulant
	ml/kg b.w.	mg tyrosine/ kg b.w.		
1	10.8	58	—	Complete incoagulability for 2 hr, 25 min. Died.
2	11.5	46	—	Complete incoagulability until death 2 hr, 56 min after injection
3	15.4	46	20	Complete incoagulability for 5 hr, 30 min. Survived.
4	12.8	106	—	Complete incoagulability until death 2 hr, 25 min after injection
5	13	82.5	25	Complete incoagulability for at least 30 min. Survived.
6	11.5	98	—	Complete incoagulability until death 3 hr after injection. Haemolysis.
7	10	61	—	Complete incoagulability until death 2 hr, 40 min after injection. Haemolysis.
8	9.4	57	—	Incomplete coagulation. Survived.
9	10	65	—	Incomplete coagulation. Survived.
10	9.2	46	—	Incomplete coagulation. Survived.
11	9.1	8.5	—	Complete coagulation. Survived.
12	10.7	86.5	25	Incomplete coagulation. Survived.

NOTE: All rabbits were injected with AFIF prepared from Armour's fraction I except for rabbits 10 and 12, which were injected with incubated human fibrinogen.

of anticoagulant, and complete incoagulability was achieved which lasted between 2 hours and 25 minutes and 5 hours and 30 minutes. The state of coagulability was tested by incubating the blood overnight at 37° C. The remaining three rabbits received less than the amount calculated as necessary for complete incoagulability to occur and the blood of these animals clotted incompletely. This was indicated by the appearance of a small fibrin clot while most of the red cells remained free and sedimented at the bottom of the tube where they formed a thick layer. The presence of clottable fibrinogen was demonstrated in these cases by diluting the serum in distilled water containing 10 m*M* calcium chloride. Five out of the seven animals in which the blood exhibited complete incoagulability died while two survived the experiment with no signs of ill effect. All three rabbits whose blood showed incomplete coagulation survived. Two of them had been injected with the anticoagulant through the marginal vein of the ear without anaesthesia and both exhibited tachypnoea and vasoconstriction of the ears which lasted for about 2 hours. The rabbits which were injected with the human incubated fraction I survived without signs of ill effect. The blood of the first animal which received the specimen incubated for 18 days clotted completely. The blood of the second rabbit injected with

the lyophilized specimen obtained from fibrinogen incubated for 29 days showed incomplete coagulation.

Two of the five animals that died exhibited gross haemoglobinaemia 2 hours after the injection of AFIF and while their blood was completely incoagulable. Autopsies performed on all five animals revealed coccidiosis of the liver. The lungs appeared congested especially around the hilar region and all the great veins of the thorax and abdomen were distended. A routine microscopic examination of the lungs, liver, and kidneys of the two animals that died with signs of haemoglobinaemia revealed congestion of the lungs with areas of heterophilic infiltration, congestion of the glomeruli of the kidneys, and areas of necrosis containing oocysts of eimeria stiedae in the congested liver. None of the animals showed signs of internal haemorrhage. Neither the surgical wound in the neck nor other incisions purposely made after the injection of AFIF showed evidence of bleeding. The bleeding time test, however, performed by cutting the marginal vein of the ear revealed a bleeding time of at least 15 minutes' duration. In four rabbits, bulldog clamps had to be applied in order to stop the bleeding. The blood of all the animals, irrespective of the degree of incoagulability achieved, remained unclotted inside the catheter used for blood sampling, while clotting was taking place prior to the injection of AFIF. The venous pressure after the injection appeared to be raised as judged by the failure of the vein to collapse during rapid aspiration of blood.

All blood samples exhibited a tendency for rapid erythrocyte sedimentation, and pronounced rouleaux formation could be seen with the naked eye. Increased sedimentation and pronounced rouleaux formation were previously observed in vitro, when human blood was mixed with human AFIF.

The coagulation studies performed included determination of the fibrinogen content, thrombin time, prothrombin time, and plasma thromboplastin generation.

Fibrinogen

Figure 1 shows the fibrinogen content before and after the injection of AFIF. Immediately after the injection a sharp rise in the fibrinogen content seems to have taken place which lasted throughout the duration of the experiment. These determinations were done by diluting the plasma in distilled water containing calcium chloride. When, however, the plasma of the rabbit injected with AFIF was diluted with normal saline instead of water, no clotting occurred even after the addition of thrombin.

In an attempt to elucidate these contradictory findings, oxalated rabbit plasma was mixed with the same volume of bovine AFIF or saline and then the clottable fibrinogen content of each mixture was determined using as diluent (1) distilled water and calcium chloride; (2) normal saline and calcium chloride; and (3) normal saline containing thrombin. The results of these determinations are shown in Fig. 2. The group on the left shows the fibrinogen content when the plasma mixtures are diluted with distilled water containing calcium chloride. The plasma–AFIF mixture seems to contain more clottable fibrinogen than the plasma–saline mixture. The reverse is true, however, when saline containing

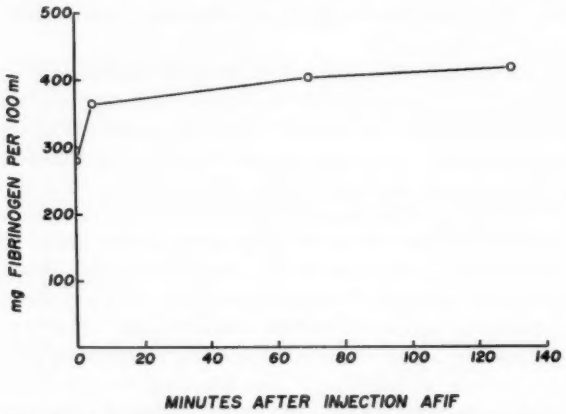


FIG. 1. Clottable fibrinogen content of plasma obtained before and after the intravenous injection of AFIF.

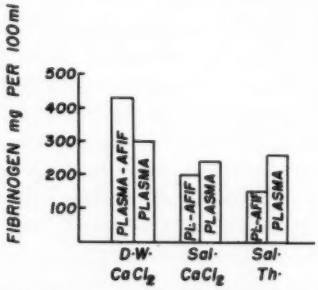


FIG. 2. Clottable fibrinogen content of plasma–AFIF and plasma–saline mixtures expressed per 100 ml plasma. Each mixture was diluted in (*a*) distilled water and calcium chloride; (*b*) saline and calcium chloride; (*c*) saline and thrombin.

calcium chloride is used as diluent. The highest clottable fibrinogen content is found in the plasma–saline mixture. When thrombin instead of calcium chloride is added to the diluent saline, the difference becomes even greater.

The following experiment elucidated the nature of these differences. One milliliter bovine AFIF was diluted in 25 ml distilled water containing 0.2 ml 1 *M* calcium chloride. The mixture was left to stand for 2 hours at room temperature at the end of which a sediment was noticed in the lower part of the beaker. Obviously, dilution of the AFIF–plasma mixture with distilled water containing calcium chloride during the determination of fibrinogen precipitates part of AFIF which gets mixed with the clot of fibrinogen and increases its protein content. In normal saline, however, AFIF does not precipitate and because it is an inhibitor of the coagulation of fibrinogen by thrombin, a lesser amount of fibrinogen clots.

Thrombin Clotting Time and One-stage Prothrombin Time

The thrombin clotting time and the one-stage prothrombin time were found of infinite length with both uncoagulable native and oxalated specimens.

Unexpectedly the thrombin time of oxalated plasmas obtained from bloods which clotted incompletely was also of infinite length. Protamine sulphate up to 250 μg per ml did not restore the coagulability of the oxalated specimens in the thrombin clotting time test. It is therefore unlikely that the state of incoagulability was due to endogenous release of heparin by the animal.

Prothrombin Time by the Adsorption and Elution Technique

Adsorption and elution of the specimens containing AFIF was introduced in order to eliminate AFIF from the system being tested. Oxalated blood obtained before and after the injection of AFIF as well as native samples withdrawn after the injection of AFIF were used for these determinations. The test was performed with blood from six rabbits, which showed complete incoagulability, and from rabbit No. 12, Table I, which was injected with human fibrinogen and whose blood partially clotted. All specimens were left at room temperature (24° C) for at least 1 hour before determination.

It can be seen from Table II that all oxalated specimens obtained after the injection of AFIF had a prothrombin time slightly longer than the ones withdrawn before the AFIF injection (about 1 second). The results with the native

TABLE II

Prothrombin time of uncoagulable blood by the adsorption and elution technique

Rabbit No.*	Prothrombin time (seconds)		
	Before injection of AFIF, oxalated	After injection of AFIF	
		Oxalated	Native
1	7.5	8.4	34.5
2	7.0	8.0	30.0
3	8.0	9.0	12.0
4	7.0	8.3	13.5
6	—	—	10.0
7	—	—	18.5
12	—	9.0	21.5

*The numbers of the rabbits correspond to those of Table I.

samples were variable. Four of them showed a markedly prolonged prothrombin time (Nos. 1, 2, and 12) while the remaining three exhibited a smaller increase as compared to the oxalated specimens. These results are at variance with results obtained in in vitro experiments with human material (2). In these, the prothrombin time of native and oxalated specimens was the same even after incubation at 37° C.

Thromboplastin Generation Test

This test was performed with oxalated plasmas and sera obtained before the administration of AFIF, and oxalated plasmas and native plasmas withdrawn after the administration of AFIF (six experiments).

The curves obtained (Fig. 3) were identical in all groups. When native plasma withdrawn after the injection of AFIF was used as supplier of both plasma and serum precursors of plasma thromboplastin, only a small amount

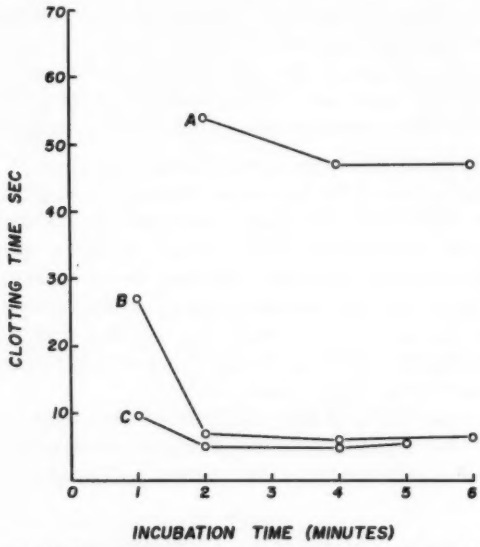


FIG. 3. Thromboplastin generation test. (A) Native plasma obtained after the injection of AFIF supplied both the plasma and the serum precursors. (B) Oxalated plasma obtained after the injection of AFIF served as supplier of the plasma precursors, and native plasma after the injection supplied the serum precursors. (C) Results obtained with plasma and serum withdrawn before the injection of AFIF.

of plasma thromboplastin was formed (line A). If, however, instead of using native plasma, oxalated plasma obtained after the injection of AFIF was used as supplier of the plasma precursors together with native plasma as supplier of the serum precursors, a normal amount of plasma thromboplastin was generated (line B). This activity was comparable to the activity generated with plasma and serum withdrawn from the same animal before the injection of AFIF (line C).

Discussion

The experiments described show that AFIF, a substance derived from fibrinogen, is as potent an anticoagulant in vivo as it is in vitro. The dose required for this effect can be accurately calculated from a test tube titration of the anticoagulant potency. The anticoagulant action is manifested immediately and under the conditions of these experiments inhibited coagulation completely for $5\frac{1}{2}$ hours.

The cause of death of the five rabbits which had received a full dose of AFIF and whose blood exhibited complete incoagulability was a multiple one. The following facts are worth noticing: (1) the two rabbits which survived, although their blood was rendered as incoagulable as the blood of the rabbits that succumbed, were transfused at the end of the experiment with 20 and 25 ml rabbit blood respectively; (2) none of the animals which died had been transfused; (3) the rabbits which received less than the amount necessary to achieve

complete incoagulability and which survived were bled of only 8–12 ml, whereas those that died were bled of 25–40 ml.[4] The only exception was the animal injected with the 29 days' incubated human fibrinogen (rabbit No. 12, Table I), which was bled of about 30 ml. This rabbit, however, was transfused with 25 ml rabbit blood immediately after the conclusion of the experiment. Prolonged Nembutal anaesthesia and coccidiosis of the liver found upon post-mortem examination were additional mortality factors. Although haemolysis had never been observed in in vitro experiments, even after incubation at 37° C, two of the rabbits which received a full dose exhibited gross haemoglobinaemia. One may speculate that the pronounced rouleaux formation acted like agglutination and consequently caused haemolysis in vivo. An immune haemolytic mechanism, however, cannot be excluded in view of the bovine origin of the injected AFIF.

The prolonged bleeding time observed after cutting the marginal vein of the ear may partly be explained on a hydrostatic etiology (increased venous pressure). It must be pointed out, however, that the surgical wound of the animal did not bleed, nor did extensions of the incision inflicted after the injection of AFIF.

The prothrombin time of native blood specimens, which had been left standing for 1 hour at 24° C before the determination, was found to be on three occasions almost as short as the prothrombin time of oxalated controls. In four other experiments, however, it was appreciably longer than the prothrombin time of the oxalated specimens. These findings indicate that the consumption of prothrombin of the shed blood of almost one half of the rabbits was inhibited in the presence of AFIF. In vitro experiments with human blood and human AFIF have demonstrated that an almost complete inhibition of the consumption of prothrombin by AFIF invariably occurs (2). No satisfactory explanation can be offered at present for the irregular occurrence of the inhibition of prothrombin consumption in the in vivo experiments.

The decrease of plasma precursors of plasma thromboplastin which was observed in all native specimens is similar in all respects to the one observed in the in vitro experiments (2). AFIF reduces the plasma precursors of plasma thromboplastin only in the presence of calcium. In the absence of calcium (oxalated specimens) no such reduction takes place. The serum precursors, however, are left intact even in the presence of calcium. It is obvious that AFIF reduces only the factors which are normally consumed during coagulation and leaves unimpaired those which are normally unaffected by coagulation (serum precursors). Furthermore, the reduction of the plasma precursors takes place under conditions which are necessary for normal coagulation to occur (presence of calcium). One may therefore speculate that the first stage of coagulation, formation of plasma thromboplastin or one of its intermediates, proceeds unimpaired in the presence of AFIF and because of this the plasma precursors are consumed. Coagulation, however, does not occur because of inhibition of subsequent stages.

[4]Body weight, 870–2380 g; mean, 1340 g.

The importance of the fluid used for the dilution of plasma during the determination of the clottable fibrinogen of blood specimens withdrawn after the injection of AFIF cannot be overemphasized if we take into consideration the opposite results obtained when distilled water or saline were used as diluents. In the presence of distilled water the values tended to be high, while in the presence of saline no signs of clottable fibrinogen could be detected. An explanation for this difference was presented earlier in this paper (Results). It must be pointed out here that the presence of circulating AFIF has not yet been investigated and pathological conditions may exist where considerable amounts of this substance may be formed in vivo (10). This endogenous AFIF could interfere with the determination of clottable fibrinogen, if saline is used as diluent. It would be advisable therefore in cases of afibrinogenaemia to confirm the lack of clottable fibrinogen by carrying out the determinations with plasma diluted in distilled water.

Acknowledgments

I wish to thank Dr. D. M. Surgenor, Harvard Medical School, Boston, Massachusetts, for supplying the human fibrinogen; Dr. F. E. Graesser, Provincial Veterinary Laboratory, for the microscopic examination of the post-mortem specimens; and Mr. L. M. Traboulay for his diligent technical assistance.

References

1. D. C. TRIANTAPHYLLOPOULOS. Proc. Western Regional Group Natl. Research Council. 11th Meeting. Banff, Alberta. January, 1957.
2. D. C. TRIANTAPHYLLOPOULOS. Can. J. Biochem. and Physiol. **36**, 249 (1958).
3. D. C. TRIANTAPHYLLOPOULOS. Am. J. Physiol. **197**, 575 (1959).
4. A. H. FREIMAN and E. E. CLIFFTON. Thrombosis et Diathesis Haemorrhagica, **2**, 269 (1958).
5. W. S. MEDART. Proc. Soc. Exptl. Biol. Med. **97**, 728 (1958).
6. W. C. TAYLOR, C. W. NASH, C. N. GILLIS, and G. L. KULLMAN. Proc. Western Regional Group Natl. Research Council. 14th Meeting. Banff, Alberta. January, 1960.
7. M. M. WINTROBE. Clinical hematology. Lea & Febiger, Philadelphia. 1956.
8. A. J. QUICK. Hemorrhagic diseases. Lea & Febiger, Philadelphia. 1957.
9. T. P. TELFER, K. W. DENSON, and D. R. WRIGHT. Brit. J. Haematology, **2**, 308 (1956).
10. D. C. TRIANTAPHYLLOPOULOS. Lancet, **1**, 1152 (1959).

WATER AND ELECTROLYTE CHANGES IN AGING PROCESS WITH SPECIAL REFERENCE TO CALCIUM AND MAGNESIUM IN CARDIAC MUSCLE[1]

KANAKA MORI[2] AND JEAN-PAUL DURUISSEAU[2]

Abstract

Concentrations of six electrolytes in seven tissues were determined in rats, the ages of which varied from 1 to 36 months, to demonstrate any changes in the electrolyte pattern during the aging process. Of the tissues studied, the electrolyte pattern of cardiac muscle showed the greatest change with age. There was a general decrease in the concentration of all cations and also of one anion, chloride, in this tissue; phosphate was the only ion to remain constant. The electrolyte pattern of the liver also changed considerably with age; the concentration of all cations decreased with the exception of calcium, which showed no variation. The electrolyte concentration of skeletal muscle changed little with age; only potassium and calcium diminished in concentration. The kidney showed an increase in the amount of calcium and phosphorus and a decrease in the level of potassium in the oldest group of animals. The aorta revealed a striking decrease in the magnesium level and a progressive increase in the amount of calcium with age. The electrolyte pattern of serum and brain was constant, except for the concentration of chloride, which was elevated in the serum of the oldest rats. The concentration of water in tissues was generally found to decrease with age.

Introduction

It is well known that the aging process is accompanied by marked alteration in the electrolyte pattern. It has also long been recognized that tissue-calcification is commonly observed in the old animal (1). Most investigations of electrolyte changes with age have been concerned mainly with calcium (2–4). Lowry *et al.* (5–7) have observed no striking changes in calcium or magnesium in the muscle, brain, liver, and kidney in a histochemical study of aging in the rat.

It has been reported that the administration of corticoids in combination with certain sodium salts can produce "infarctoid cardiac necroses", even in very young rats (8–10). These necroses are accompanied by extensive changes in concentrations of calcium and magnesium, with the latter decreasing specifically in the necrotic heart (11). It is often observed clinically that old people are more prone to degenerative cardiac diseases than the young. These various findings suggest that the electrolytes, especially calcium and magnesium, are altered significantly in aging. The present paper deals with the determination of water and six electrolytes in seven tissues of normal rats, the ages of which varied from 1 to 36 months.

Methods and Materials

The animals used were female Sprague–Dawley rats, and they were fed Purina Fox Chow and water ad libitum. They were divided into four groups

[1]Manuscript received in original form June 9, 1959, and, as revised, December 21, 1959.
Contribution from Laboratoire de Biochimie, Institut de Médecine et chirurgie expérimentales, Université de Montréal, Montreal, Que.
[2]Present address: Institut de Gérontologie, Hôpital Notre-Dame de la Merci, 555 Gouin Blvd. West, Montreal.

according to their age, as follows: group I, 13 rats, mean age 1.1 (0.9–1.2) months; group II, 15 rats, mean age 1.6 (1.5–2.0) months; group III, 13 rats, mean age 11.6 (10–12) months; and group IV, 6 rats, mean age 35.4 (34–36) months. Food but not water was removed for 18 hours before sacrifice and autopsy. At the time of autopsy, the organs under investigation were examined with the aid of a dissecting magnifying glass, and any rat with a sign of abnormality was discarded.

Serum, brain, abdominal muscle, cardiac muscle, liver, kidney, and aorta were analyzed for electrolyte concentrations. Serum was obtained as described previously (11). Samples for the analysis of the other tissues were taken immediately after the collection of blood, dried, defatted with ether:petroleum ether (1:1) mixture, and prepared for the analysis of electrolytes according to the method of Lowry and Hastings (5). Thoracic and abdominal aortae were removed and dissected free of adventitia, then prepared similarly.

Sodium, potassium, and total phosphorus were measured by the same methods described in a previous publication (11). Calcium was determined by a method devised by one of us (12); magnesium, by the method of Orange and Rhein (13); and chloride, by Lowry and Hastings (5). All electrolyte values of the tissues are expressed in units per kg of dry, fat-free solids. We have assumed that chloride is entirely extracellular (14), therefore, extracellular water in muscle is considered to be the volume of distribution of chloride in muscle. Intracellular water was calculated as the difference between the total and extracellular water content of the muscle. The extracellular concentration of each cation in muscle was also calculated from the concentration of each cation in serum water by correcting for the Gibbs–Donnan effect, and the intracellular cation concentration was calculated from the volume of intracellular water and the difference between the amounts of total and extracellular cation associated with 1 kg dry, fat-free solids. These concentrations are expressed in units (meq) per kg of the respective cellular water. The system of calculation was based on that described by Manery (14) and was as follows:

(1) extracellular water (ECW) $= \dfrac{(\mathrm{Cl})_t \times 980}{[\mathrm{Cl}]_s}$, g/kg of dry, fat-free solids,

(2) intracellular water (ICW) = total water − ECW, g/kg of dry, fat-free solids,

(3) extracellular cation $(\mathrm{cation})_e = \dfrac{[\mathrm{cation}]_s \times r \times \mathrm{ECW}}{1000}$, meq/kg of dry, fat-free solids,

(4) intracellular cation $(\mathrm{cation})_c$ = (total cation)$_t$ − $(\mathrm{cation})_e$, meq/kg of dry, fat-free solids,

(5) concentration of cation in extracellular fluid = $[\mathrm{cation}]_s \times r$, meq/kg of extracellular water,

(6) concentration of cation in intracellular fluid $= \dfrac{(\mathrm{cation})_c}{\mathrm{ICW}} \times 1000$, meq/kg of intracellular water,

in which brackets $[\,]_s$ denote the concentration in meq per kg of serum water,

parentheses $(\,)_t$ the content in meq per kg of dry and fat-free solids. The factor r corrects for the difference between plasma water and interstitial fluid concentration of each cation and chloride due to the Donnan effect, and the value of r for each cation was based upon the one described by Conway (15).

Results

Serum

The chloride concentration in the serum of the oldest group (group IV) was significantly higher ($P<0.001$) than in any other group (Table I).

Brain

The concentrations of water and electrolyte in the brain did not show any important variation, in all the groups tested (Table I).

Skeletal Muscle

Although there were great differences in age between the various groups of animals (1 to 36 months), no important variations in the concentrations of electrolytes could be detected, except in that of calcium (Table II). The concentration of this electrolyte decreases with age ($P<0.01$). This variation in calcium is accompanied by a parallel decrease in the water level ($P<0.01$), especially in the extracellular water level. The intracellular concentration of sodium in the rats of the oldest group is slightly higher than that in the other groups; on the other hand, the intracellular concentration of potassium is remarkably decreased in the oldest group.

Cardiac Muscle

Of all the tissues tested, cardiac muscle is the most profoundly affected by age, as far as the electrolyte pattern is concerned. All the cations studied decreased in the rats of the oldest group (Table III). Only one anion, phosphate, did not change. This over-all decrease in the concentrations of electrolytes is not due to an increased water content since the cardiac muscle in group IV showed the lowest water level. The constancy of the phosphorus level also supports this view. In other words, it appears that there is a general reduction in the amount of electrolytes in the old cardiac muscle (sodium, $P<0.01$; potassium, $P<0.01$; calcium, $P<0.02$; magnesium, $P<0.001$, and chloride, $P<0.01$). The intracellular concentration of cation was also markedly decreased in this tissue.

Liver

Levels of all cations measured in the liver of the oldest group were significantly decreased with the exception of calcium, which remained constant (sodium, $P<0.001$; potassium, $P<0.001$; and magnesium, $P<0.001$) (Table IV). However, although statistically not significant, there seems to be a progressive decrease in the level of calcium in the liver, paralleled by a similar variation in the water level, a change similar to that described for skeletal muscle.

Kidney

A remarkable decrease in the concentration of potassium was observed in the kidney of the oldest rats in group IV ($P<0.001$) (Table IV). There was

TABLE I

Water and electrolytes of serum and brain in rats at various ages*

	Serum†							Brain‡						
Group	H_2O, g	Na, meq	K, meq	Ca, meq	Mg, meq	Cl, meq	P, mmole	H_2O, g	Na, meq	K, meq	Ca, meq	Mg, meq	Cl, meq	P, mmole
I (1.1 mon.)	951 ±5	165.5 ±3.7	6.03 ±.26	6.25 ±.36	2.41 ±.46	109.1 ±1.2	3.64 ±.31	3461 ±45	220 ±5	423 ± 9	26.8 ± .8	58.3 ±3.6	156 ±8	360 ±19
II (1.6 mon.)	944 ±6	162.0 ±2.9	6.37 ±.14	6.16 ±.20	2.66 ±.39	112.7 ±1.1	3.54 ±.27	3285 ±67	212 ±3	413 ±10	24.9 ± .8	58.2 ±3.0	149 ±6	386 ±10
III (11.6 mon.)	947 ±6	156.8 ±3.7	6.18 ±.24	6.04 ±.26	2.55 ±.52	110.0 ±1.4	3.24 ±.28	3318 ±53	215 ±4	412 ± 8	25.5 ± .7	54.0 ±3.5	145 ±7	388 ±11
IV (35.4 mon.)	940 ±7	148.9 ±3.8	5.82 ±.29	5.40 ±.41	2.66 ±.58	121.1 ±0.9	3.82 ±.42	3165 ±71	205 ±4	382 ±10	25.4 ±1.3	53.8 ±2.5	128 ±9	380 ±21

*All values are expressed as the mean ± standard error.
†Values are shown in g, meq, or mmole per kg (or l.) of serum water.
‡Values are shown in g, meq, or mmole per kg of dry, fat-free solids.

TABLE II

Distribution of water and electrolytes of skeletal muscle in rats at various ages

Group	Original data*							Derived data*							
	H_2O, g	Na, meq	K, meq	Ca, meq	Mg, meq	Cl, meq	P, mmole	ECW, g	ICW, g	Na_e, meq	Na_c, meq	K_c, meq	Ca_c, meq	Mg_c, meq	TIC, meq
I (1.1 mon.)	3608 ±62	195 ±8	551 ±32	27.0 ±1.6	87.8 ±4.5	64.7 ±4.9	295 ±18	581	3027	156	34	181	8	29	252
II (1.6 mon.)	3518 ±39	186 ±5	540 ±34	25.8 ±1.4	94.2 ±5.0	61.8 ±4.6	324 ±17	537	2981	152	35	180	8	31	254
III (11.6 mon.)	3479 ±54	178 ±9	580 ±36	22.0 ±1.2	91.0 ±6.6	63.5 ±3.8	296 ± 9	566	2913	147	33	197	7	31	264
IV (35.4 mon.)	3301 ±42	184 ±13	477 ±39	19.9 ±1.3	84.1 ±7.6	52.4 ±3.1	313 ±16	424	2877	140	43	165	6	29	243

*Values are expressed as the mean in g, meq, or mmole per kg of dry, fat-free tissue ± standard error.

†ECW = extracellur water, ICW = intracellular water, both are given in g/kg of dry and fat-free solids. Na_e = concentration of sodium in extracellular water, Na_c = concentration of sodium in intracellular water, etc., TIC = total cation concentration in intracellular water; these values are all given in meq/kg (or l.) of the corresponding cellular water. For example of calculation, see text.

TABLE III

Distribution of water and electrolytes of cardiac muscle in rats at various ages

	Original data*							Derived data†							
Group	H_2O, g	Na, meq	K, meq	Ca, meq	Mg, meq	Cl, meq	P, mmole	ECW, g	ICW, g	Na_e, meq	Na_c, meq	K_c, meq	Ca_c, meq	Mg_c, meq	TIC, meq
I (1.1 mon.)	3701 ±50	230 ±10	385 ±16	41.2 ±3.9	86.4 ±7.6	173 ±8	335 ±14	1554	2147	156	—	176	16	39	231
II (1.6 mon.)	3682 ±61	247 ±8	396 ±12	30.7 ±1.9	82.7 ±5.9	175 ±9	344 ± 9	1522	2160	152	7	179	11	37	234
III (11.6 mon.)	3549 ±45	190 ±9	389 ±15	25.5 ±2.0	68.8 ±5.7	167 ±7	348 ±11	1488	2061	147	—	184	9	32	225
IV (35.4 mon.)	3382 ±95	112 ±5	355 ±14	21.2 ±1.4	47.4 ±4.5	141 ±6	339 ± 8	1141	2241	140	—	156	7	20	183

*Values are expressed as the mean in g, meq, or mmole per kg of dry, fat-free tissue ± standard error.

†ECW = extracellular water, ICW = intracellular water, both are given in g/kg of dry and fat-free solids. Na_e = concentration of sodium in extracellular water, Na_c = concentration of sodium in intracellular water, etc., TIC = total cation concentration in intracellular water; these values are all given in meq/kg (or l.) of the corresponding cellular water. For example of calculation, see text.

also an important increase in the amount of calcium ($P<0.001$) and phosphorus ($P<0.01$) in the kidney of the same group.

Aorta

A striking decrease in the level of magnesium in this tissue ($P<0.001$) could be seen in the oldest group (Table IV). There was also a progressive increase in the amount of calcium (group III, $P<0.02$; group IV, $P<0.01$) accompanied by a gradual decrease in the level of water (group III, $P<0.02$; group IV, $P<0.001$), as the animals grew older.

TABLE IV

Water and electrolytes of liver, kidney, and aorta in rats at various ages*

Tissue	Group	H_2O, g	Na, meq	K, meq	Ca, meq	Mg, meq	Cl, meq	P, mmole
Liver	I (1.1 mon.)	3211 ±75	166 ± 8	423 ±11	18.1 ±2.1	151.0 ±11.8	140 ±12	428 ±29
	II (1.6 mon.)	3086 ±72	171 ± 9	414 ±17	18.4 ±1.6	140.9 ± 8.6	137 ± 8	418 ±31
	III (11.6 mon.)	2904 ±87	148 ±11	405 ±12	17.2 ±1.6	117.2 ± 7.4	132 ± 9	422 ±44
	IV (35.4 mon.)	2885 ±49	118 ± 5	347 ± 7	15.9 ±1.8	57.5 ± 4.0	128 ± 6	446 ±36
Kidney	I (1.1 mon.)	3682 ±92	319 ±23	347 ±16	30.3 ±2.5	69.3 ± 8.0	357 ±13	385 ±12
	II (1.6 mon.)	3589 ±51	324 ±15	327 ±17	25.4 ±2.7	69.2 ± 5.5	360 ±21	418 ±14
	III (11.6 mon.)	3564 ±39	306 ±12	311 ±17	28.4 ±1.6	71.6 ± 4.1	346 ±19	497 ±24
	IV (35.4 mon.)	3573 ±69	301 ±18	251 ±12	40.4 ±2.7	62.0 ± 3.9	331 ±26	543 ±37
Aorta	I (1.1 mon.)	2516 ±68	389 ±21	162 ± 9	142 ±13	83.3 ± 5.6	235 ±10	78 ± 6
	II (1.6 mon.)	2457 ±28	399 ±19	143 ±12	141 ±10	72.8 ± 9.1	241 ±13	84 ± 7
	III (11.6 mon.)	2335 ±29	444 ±23	173 ±18	177 ± 5	100.8 ± 9.1	232 ± 9	78 ± 5
	IV (35.4 mon.)	2213 ±50	405 ±19	157 ±10	195 ±13	41.5 ± 1.8	210 ±12	93 ± 8

*All values are expressed as the mean in g, meq, or mmole per kg of dry, fat-free tissue ± standard error.

Discussion

It has been reported that in man the amounts of water and chloride are higher in the newborn than in the adult (both components are expressed in units per kg fresh body weight) and that the higher water level in the body of the newborn is due to a greater portion of extracellular fluid (16). If computed in terms of dry, fat-free body solids, the amounts of water in the newborn and in the adult will be about 4900 g/kg unit and 2700 g/kg unit, respectively; and the amounts of chloride will be about 255 mmoles/kg unit and 152 mmoles/kg unit, respectively. And out of about 4900 g of water per kg of dry, fat-free body solids in the newborn, about 2400 g of water are contained in the predominantly extracellular phase; on the other hand, in the adult this amount is about 900 g. This tendency is also observed in the present studies in all tissues except kidney. Therefore, it would appear that the extracellular phase is smaller and

drier in older than in younger animals. The decrease in the water level of the aged aorta has also been reported in human (17). In the skeletal muscle of the oldest rats, water was reduced in both extra- and intra-cellular spaces (Table II). On the other hand, only extracellular water was remarkably decreased in the cardiac muscle of the rats in the same group. There was also a decrease in the intracellular concentration of potassium, calcium, and magnesium in aged hearts. This lowering of the intracellular concentration of cations cannot be ascribed to an increase in intracellular water, because no such increase was observed (Table III). These changes in the water and electrolytes of cardiac muscle with age may be important to the understanding of various gerontological problems.

The present results (Table I) support the view that the concentration of electrolyte in the brain appears to be quite independent of age as observed by Lowry *et al.* (7), although Streicher (18) recently reported that the concentration of calcium in the brain of younger rats is statistically lower than that of aged rats. However, the post-mortem artifacts of the brain are so numerous that it can not be said that we have here conflicting evidence.

The concentration of potassium decreased in the skeletal and cardiac muscle, liver, and kidney of aged rats. These decreases were associated with decreases in the level of magnesium in the cardiac muscle and liver. Potassium is known to play an important role in the normal metabolism of pyruvate (19), and it is also well known that magnesium is essential as a cofactor for various enzyme systems. Therefore, the decrease in both of these intracellular cations in aged heart muscle and liver might be connected with a general reduction in cellular functions of these tissues. Although some workers could not detect significant changes in DNA and RNA of the liver in aged mice (20), several others have found a remarkable decrease in phosphocreatine level (21, 22) and metabolic rate (23) of the aged heart in rats. The decreased intracellular concentration of potassium in aged skeletal muscle and the decreased amount of potassium in aged kidney may also indicate some dysfunction in these tissues. The hyperchloremia observed in the aged rats may in part be due to a reduction in renal chloride excretion. Renal dysfunction may result from an alteration in adrenocortical functions with age (24).

It has been reported that the plasma calcium concentration in very old men (80 years old) is lower than in younger (25), and a similar tendency is apparent in the present experiments (Table I). A general tendency to a shift of calcium from bones to soft tissues with age has been described by Bazilewitch and Pravdena (26), and the increase in the calcium level of the kidney and aorta of aged animals found in the present studies has also been observed by Vermooten (27) and Haythorn *et al.* (28). However, other investigators have not observed such a shift of calcium (7). It is of interest that the calcium level of heart in the oldest rats is much lower than in the youngest ones (Table III), though calcium has been reported to increase in the aged human heart (29). Besides, the intracellular concentration of calcium in the aged heart was decreased in the present studies. There is evidence that nucleic acids are intimately associated with calcium and magnesium and that these bivalent cations are closely linked with

cellular structures and functions (30). It is possible that calcification observed under the microscope does not indicate the true status of calcium in cytoplasm because of the limitation of the usual techniques employed in such studies. Lansing *et al.* (31) observed that arterial elastic fibers, which in youth were entirely mineral free, with advancing age took up progressively greater amounts of calcium and that, of the amino acid components of elastic fibers, both aspartic and glutamic acids showed a sharp increase with age. They concluded that the greater amounts of these dicarboxylic amino acids might account for the increased affinity for calcium.

The remarkable decrease in the amount of magnesium in the aged aorta found in the present experiments has also been reported, in man, by Rechenberger and Hevelke (32). The fact that total phosphorus in the aged heart and liver remained constant is in accordance with earlier reports (20, 22).

Acknowledgments

The authors are greatly indebted to Dr. E. Robillard, Professor of Physiology, University of Montreal, for his encouragements and for supplying the 36-month-old rats used in the present experiments, and to Mr. and Mrs. A. Andrasko for their technical help in the course of these studies.

This work was supported by a grant from the Gustavus and Louise Pfeiffer Research Foundation and by Grant No. H-3688 from the National Heart Institute (U.S. Public Health Service).

References

1. C. M. McCay, G. Sperling, and L. L. Barness. Arch. Biochem. **2**, 469 (1943).
2. M. D. Bogonoff, N. W. Shock, and M. P. Nicholas. J. Gerontol. **8**, 272 (1953).
3. D. M. Hegsted, I. Moscoso, and C. J. I. Collazos. J. Nutrition, **46**, 181 (1952).
4. E. C. Owen. Biochem. J. **33**, 22 (1939).
5. O. H. Lowry and A. B. Hastings. J. Biol. Chem. **143**, 257 (1942).
6. O. H. Lowry, A. B. Hastings, T. Z. Hull, and A. N. Brown. J. Biol. Chem. **143**, 271 (1942).
7. O. H. Lowry, A. B. Hastings, C. M. McCay, and A. N. Brown. J. Gerontol. **1**, 345 (1946).
8. H. Selye. Can. J. Biochem. and Physiol. **36**, 717 (1958).
9. H. Selye. Angiology, **9**, 262 (1958).
10. H. Selye, S. Renaud, and M. Nadasdi. J. Pharmacol. Exptl. Therap. **122**, 504 (1958).
11. J. P. Duruisseau and K. Mori. Brit. J. Exptl. Pathol. **60**, 250 (1959).
12. K. Mori. Arch. Biochem. Biophys. **83**, 552 (1959).
13. M. Orange and H. C. Rhein. J. Biol. Chem. **189**, 379 (1951).
14. J. F. Manery. Physiol. Revs. **34**, 334 (1954).
15. E. J. Conway. Physiol. Revs. **37**, 84 (1957).
16. D. C. Darrow and S. Hellerstein. Physiol. Revs. **38**, 114 (1958).
17. V. C. Myers and W. W. Lang. J. Gerontol. **1**, 441 (1946).
18. E. Streicher. J. Gerontol. **13**, 356 (1958).
19. H. A. Lardy and J. A. Ziegler. J. Biol. Chem. **159**, 343 (1945).
20. D. M. Schulz. Arch. Biochem. Biophys. **27**, 57 (1950).
21. C. G. Casten. Am. Heart J. **39**, 353 (1950).
22. C. G. Casten and M. F. Mason. J. Clin. Invest. **26**, 1177 (1947).
23. J. Jehl and A. Wollenberger. Federation Proc. **9**, 288 (1950).
24. R. E. Weston. Hormones and the aging process. *Edited by* E. T. Engle and G. Pincus. Academic Press, Inc., New York. 1956. p. 201.
25. E. M. Greisheimer, O. H. Johnson, and M. Ryan. Am. J. Med. Sci. **177**, 704 (1929).
26. I. V. Bazilewitch and L. I. Pravdena. J. méd. Ukraine, **10**, 1267 (1940).
27. V. Vermooten. J. Urol. **48**, 27 (1942).
28. S. R. Haythorn, F. A. Taylor, H. W. Crago, and A. Z. Burrier. Am. J. Pathol. **12**, 283 (1936).

29. L. L. BARNES. Am. J. Pathol. **18**, 41 (1941).
30. L. MONNÉ. Advances in Enzymol. **8**, 1 (1948).
31. A. I. LANSING, M. ALEX, and T. B. ROSENTHAL. J. Gerontol. **5**, 112 (1950).
32. J. RECHENBERGER and G. HEVELKE. Z. Alterforsch. **9**, 309 (1956).

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